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# Investigations Concerning Hydrolysis and Stabilization of Antiradiation Compounds

Annual Report

D. J. Mangold, Ph.D., Principal Investigator
N. F. Swynnerton, Ph.D.
C. W. Lew, B.S.
E. M. Gause, M.S.

January 1982

Supported by
U. S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701

Contract DAMD17-80-C-0128

Southwest Research Institute
6220 Culebra Road
P. O. Drawer 28510
San Antonio, Texas 78284

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An'iradiation compounds: WR 2721; hydrolytic and thermal properties; encapsulation; drug dosage forms.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This report contains results of the following studies:

1. Hydrolysis of WR 2721 various pH levels.
2. Encapsulation of WR 2721 for use as oral dosage forms.
3. In vitro evaluation of the most promising samples.
4. Development of analytical methodology for the active species.

AD_____

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## SUMMARY

This report covers studies conducted on S-2-(3-aminopropylamino)-ethylphosphorothioic acid, WR 2721, over the period January 16, 1981 through January 15, 1982, which includes portions of the first and second years of the contract. The most significant results obtained during this report period are the following.

1) Unencapsulated WR 2721 was found to be hydrolytically stable over the pH range of 7.4 to 11.0.

2) WR 2721 was successfully encapsulated in certain matrices such that the WR 2721 was not significantly hydrolyzed in 1.5 hours at 37°C in buffered solutions of pH 1.0 or 3.0.

3) The more promising microspheres and microcapsules released the WR 2721 within two hours at pH 7.5 in buffered solutions.

4) Analytical procedures were developed for:

   - WR 2721 (directly) in microcapsules using an HPLC procedure;
   - WR 2721 (directly) in buffered solution (pH 7.4) using an HPLC procedure;
   - WR 2721 (indirectly) in synthetic intestinal fluid using an HPLC procedure based upon a derivatization of WR 2721 with o-phthalaldehyde.

5) An analytical procedure is being investigated for the analysis of blood plasma for WR 2721 and its mercaptan and disulfide. At present, it appears that a method based on derivatizing the species with fluorescamine is most promising.

## FOREWARD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW Publication No. (NIH) 78-23, Revised 1978].

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# I. STATEMENT OF PROBLEM

Over a period of several years, the U.S. Army Medical Research and Development Command has been actively pursuing the development of a drug or combination of drugs which could be taken by military personnel for protection from the effects of the ionizing radiations from a nuclear weapons attack. Several chemical compounds (when dosed intravenously in animal studies) were found to be promising, in particular the phosphorothioates. The best of these materials, S-2-(3-aminopropylamino)ethylphosphorothioic acid, WR 2721, has been shown to protect mice, dogs, and Rhesus monkeys against X-ray or gamma radiation and to protect mice against neutron radiation. However, oral dosing of WR 2721 has failed to protect either dogs or monkeys. With dogs, oral dosing of WR 2721 produced vomiting.

In an attempt to explain the lack of activity following oral administration, it has been postulated that WR 2721 is readily hydrolyzed to the mercaptan in the stomach of the animal species and that the mercaptan is poorly absorbed. (Some credence has been given to this hypothesis as a result of the studies during the first year of this program which have shown that WR 2721 is readily hydrolyzed at a pH of 1.0.) If such be the case, then it would be highly appropriate to protect WR 2721 with an enteric coating for passage through the stomach. One means of applying such a coating would be by the use of microencapsulation processes which, in some cases, may subject the drug to elevated temperatures. Therefore, prior to undertaking any microencapsulation studies, adequate thermal stability of WR 2721 had to be established, and this was shown by heating WR 2721 at 60°C for one hour without any measurable decomposition.

During the present term of the contract, WR 2721 has been successfully encapsulated as microspheres or microcapsules using several different matrices which will protect it from acid hydrolysis yet will release it in solutions of pH 7.5. Further studies are in progress to improve and optimize these encapsulation formulations.

It has been proposed to conduct in vivo evaluations of the most promising formulations using the beagle dog as the animal model. These studies will require the development of analytical procedures for the determination of WR 2721, its mercaptan, and disulfide in blood serum.

## II. BACKGROUND

During the period covered by the first annual report under this contract, it was demonstrated that:

1. WR 2721 is rapidly hydrolyzed at pH 1.0;

2. WR 2721 is thermally stable up to one hour at 60°C under nitrogen.

These results justified the approach proposed for the development of an acceptable oral dosage form of the drug by encapsulation with enteric-type coatings which would protect the drug from acid hydrolysis during passage through the stomach. The thermal stability of WR 2721 is of importance since elevated temperatures are involved during the proposed encapsulation process.

Therefore, the studies conducted during the period covered by this second annual report were involved with the encapsulation of WR 2721, evaluation of the encapsulated product, and development of analytical procedures for the determination of WR 2721 and some of its decomposition products in various media.

# III. EXPERIMENTAL

## A. Microencapsulation

### 1. Materials

#### a. Description and Source

All materials used in the encapsulation formulations were standard food-grade, and their sources are listed in Appendix A.

#### b. Purity

The glycerides, fatty acids, fatty alcohols, and paraffin wax listed in Appendix A were used as received.

WR 2721 was obtained as the trihydrate from WRAIR and was used as received except, as noted, where the particle size was reduced by milling with a mortar and pestle. The material, as originally received, was labeled "WR 2721 AU BJ 09506AJ-68-2." Analysis was reported as 79.2 percent WR 2721, <0.5 percent mercaptan and disulfide, 19.7 percent $H_2O$ and approximately 0.4 percent unidentified impurities.

### 2. Procedures

The two-orifice SwRI centrifugal extrusion head (Figure 1), driven by a variable speed DC motor (not shown), was used to prepare the microcapsules and microspheres. For microcapsule production, shell and fill materials were fed through concentric tubes which enter the top of the rotating head through a bushing and an O-ring seal. Desired feed rates of the shell and fill materials were maintained by Zenith pumps attached to variable speed drives. For the production of microspheres, the equipment was modified to eliminate the feed (shell material) to the outer orifice.

A hot-melt system was used to prepare microcapsules and microspheres. All reservoir, pump and feed lines were separately wrapped with heating tapes individually controlled by variable transformers. Head temperature was controlled by a Lepel high frequency induction heater. Nozzle size, head rotational speed, and feed rates were optimized to produce microcapsules and microspheres mainly in the 500 to 710 μm size range. Final sizing was done using standard stainless steel sieves.

### 3. Evaluation

#### a. Hydrolytic Stability

Hydrolytic stability of microspheres and microcapsules was determined by subjecting samples to HCl/KCl buffer solutions at pH levels of approximately 1 and 3 at 37°C for 90 min using the standard rotating bottle method (National Formulary XIII). The procedure is described in detail in Appendix B.

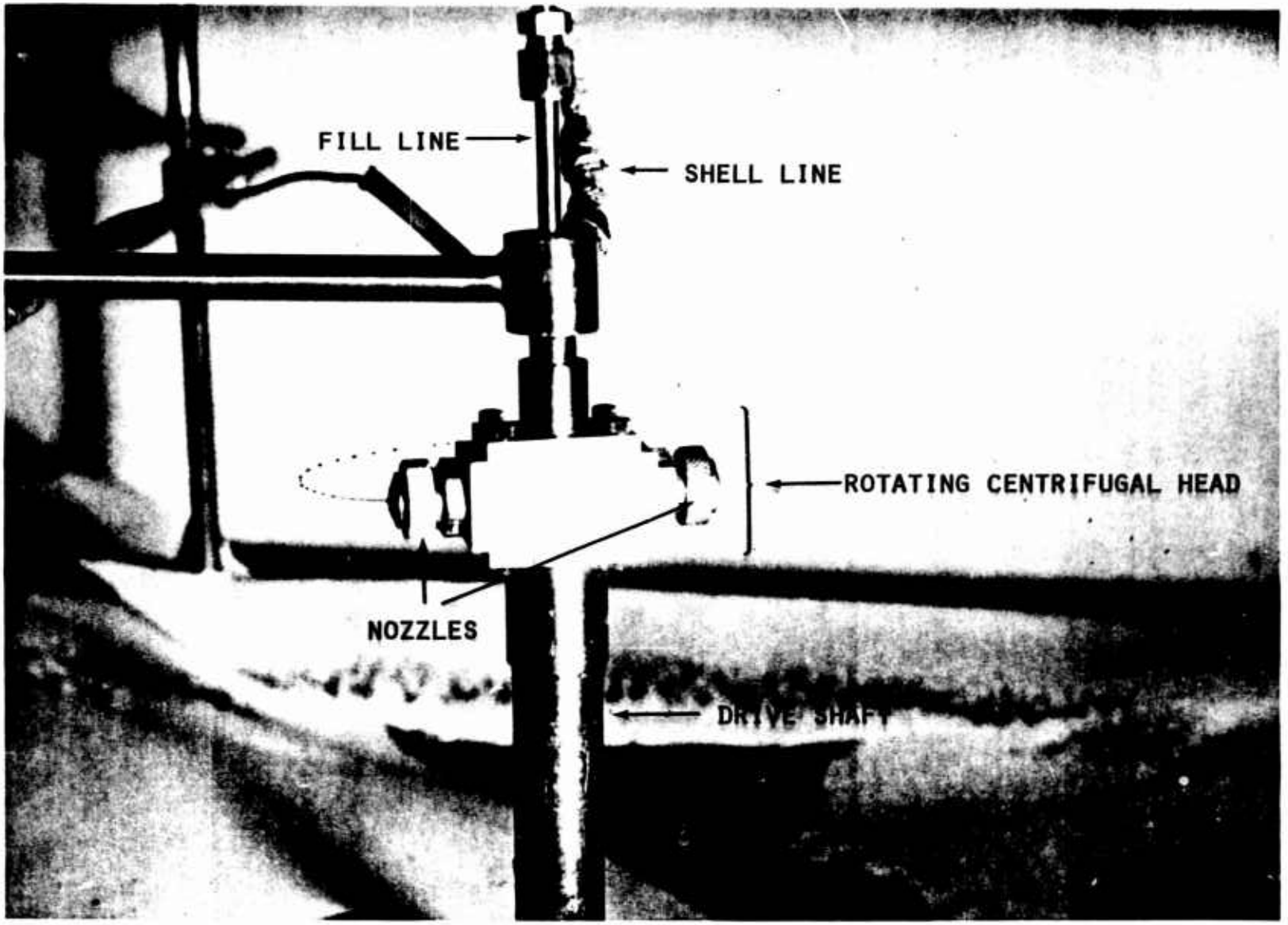


Figure 1. Two-Nozzle Centrifugal Extrusion Head with One Nozzle Plugged

b. In Vitro Release Rates

Release rates of microspheres and microcapsules were determined by subjecting samples to Tris buffer [aqueous solution of HCl/tris(hydroxymethyl)aminomethane] and to synthetic intestinal fluid at approximately pH 7.5 at 37°C for periods 0.25, 0.50, 1, 2, 3, 5, and 8 hours using the standard rotating bottle method (National Formulary XIII). The procedure is described in detail in Appendix C.

## B. Hydrolytic Stability of Unencapsulated WR 2721

Procedures for determination of the hydrolytic stability of unencapsulated WR 2721 at basic pH were the same as those used for the studies at acidic pH and have been previously described.[1,2] In one experiment, a direct HPLC procedure was used to determine WR 2721 levels as a check on the principle method of analysis. This HPLC procedure is described below in the Analytical Procedures section of this report.

## C. Analytical Procedures

### 1. Development of Methods for In Vitro Studies (SwRI)

A simple, rapid analysis for WR 2721-containing microspheres and microcapsules has been developed. The method involves dissolution of the microsphere and microcapsule shell and/or matrix components in an organic solvent, extraction of WR 2721 into water, and direct injection of the aqueous solution onto an HPLC column. Detection is by UV at 205 nm. Potential interferences such as matrix materials and WR 2721 decomposition products are either 1) separated during sample preparation or on the HPLC column or 2) have no UV absorption at the detection wavelength. High precision has been demonstrated for this method (Table I). Analysis of seven samples taken from a single microsphere production run gave a relative standard deviation (RSD) of 1.1%. This also speaks well for the uniformity of the microspheres. The procedure and representative chromatograms are presented in Appendix D. However, these conditions for the assay of WR 2721-containing microspheres were unsuitable for the analysis of the drug present in synthetic intestinal fluid (in vitro release-rate studies). Substances present in the synthetic intestinal fluid co-elute with WR 2721, and no modifications to the existing procedure were found to solve this problem. Conversations with the Waters and Associates applications laboratory personnel led to the adaptation of a modified amino acid HPLC analysis to the analysis of WR 2721. The method involves precolumn derivatization of WR 2721 with o-phthalaldehyde (OPA) and subsequent analysis on a reverse-phase C-18 column with fluorescence detection. This method is similar to that related to SwRI by Dr. Nesbitt Brown of WRAIR. Ethanethiol has been substituted for mercaptoethanol, and the proportions of reagents have been varied somewhat. The net effect was to increase the stability of the OPA derivative, but the derivative still decomposes at an appreciable rate. Using automated repeat injections from the same vial of derivatized WR 2721, a reduction in peak area with time is

observed. Figure 2 is a plot of ln (peak area) versus time for this experiment. Assuming first-order kinetics, the rate constant for the decomposition was $1.52 \times 10^{-2}$ $min^{-1}$ ($t_{1/2}$ = 46 min). The method is usable, despite the instability of the derivative, provided that derivatization reaction times are kept constant. To demonstrate precision, replicate samples of WR 2721-containing microspheres were dispersed in chloroform, the WR 2721 was extracted into water and the aqueous solutions were derivatized and analyzed by reverse-phase HPLC. The data are given in Table II. Relative standard deviation for six replicate analyses was 4.3%. This method is less precise than the direct HPLC method described above which uses UV detection of WR 2721 at 205 nm. The procedure and representative chromatograms are presented in Appendix E.

Samples of the mercaptan and the disulfide of WR 2721 were treated with the OPA reagent and analyzed under the same HPLC conditions as the WR 2721 derivative. Much longer k' values were observed for these compounds and a "stronger" mobile phase was necessary to obtain good peak shapes and reasonable elution times. Optimization of HPLC conditions for analysis of the mercaptan and the disulfide has not yet been carried out.

2. Development of Methods for In Vivo Studies (SFRE)

a. Objectives

The primary objective of this phase is the development of assay methodology suitable for quantitative estimation of the drug WR 2721 in plasma in the microgram/milliliter range. Secondary objectives are the simultaneous measurement of metabolite forms of the drug, and determination of free versus bound forms of the drug.

The development of this specific assay is complicated by the chemical, biochemical, and physical properties of the drug, i.e., it is a small molecule; water-soluble, but susceptible to hydrolysis; not appreciably soluble (or stable) in organic solvents; does not possess distinctive spectrophotometric or fluorometric properties; and contains multiple functional groupings which mimic endogenous biochemical molecules such as amino acids, phosphate esters, thiols, and disulfides. These factors must obviously all be accommodated in the ultimate analytical scheme.

b. Technical Approach

A survey of the biochemical and pharmacological literature relevant to the assay of drugs in biological fluids was initially undertaken. Based on the results of this survey, a generalized experimental approach to the design of an assay was outlined as follows:

1. Assay end-step: Chromatographic method, probably HPLC. This should allow separation of unchanged drug from plasma components and from drug metabolites.

TABLE I. PRECISION OF ANALYSIS OF WR 2721 MICROSPHERES USING HPLC WITH DETECTION AT 205 nm

| Sample Number | Weight Percent WR 2721 |
|---|---|
| 1 | 14.9 |
| 2 | 14.7 |
| 3 | 14.5 |
| 4 | 14.7 |
| 5 | 14.6 |
| 6 | 14.5 |
| 7 | 14.4 |

$\bar{x}$ = 14.6
SD = 0.17
RSD = 1.1%

TABLE II. PRECISION OF ANALYSIS OF WR 2721 MICROSPHERES USING HPLC WITH OPA DERIVATIZATION

| Sample Number | Weight Percent WR 2721 |
|---|---|
| 1 | 17.2 |
| 2 | 16.2 |
| 3 | 15.2 |
| 4 | 15.8 |
| 5 | 15.6 |
| 6 | 16.1 |

$\bar{x}$ = 16.0
SD = 0.68
RSD = 4.3%

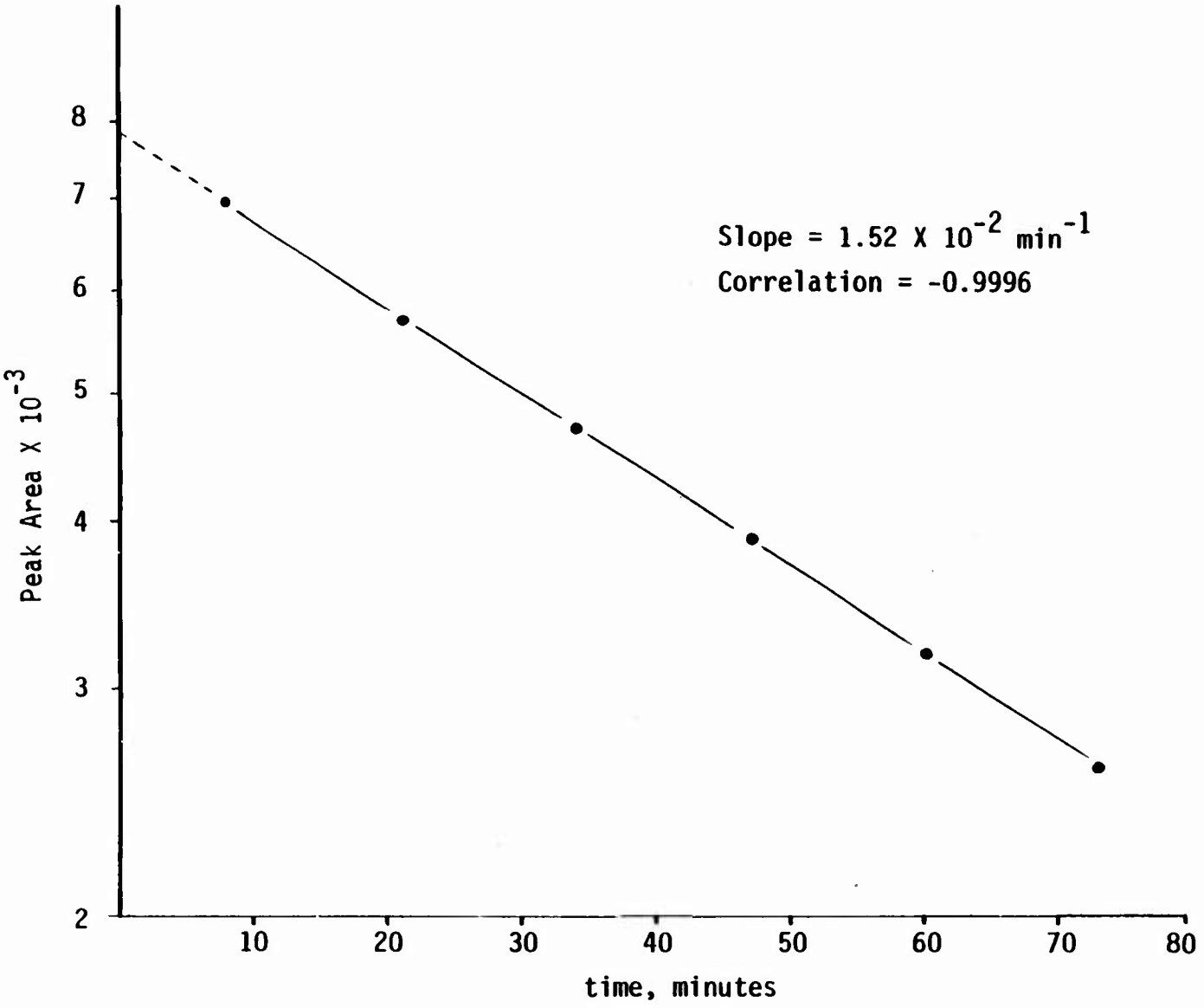


Figure 2. Decomposition Rate of OPA Derivative of WR 2721

2. Detection: Derivatization to obtain chromophore or fluorophore. Derivatization reaction should involve free amino group, or $[H_2N(CH_2)_3NH-]$ moiety, so that metabolite forms as well as drug itself would be derivatized.

3. Sample work-up: (a) Solvent extraction or precolumn cleanup and/or (b) deproteinization of plasma before or after derivatization.

4. Anticipated problems: Accuracy (i.e., specificity) or ability to separate out contributions from plasma constituents; instability of WR 2721 with respect to chemical hydrolysis, enzymatic hydrolysis (acid phosphatase); significant loss of drug due to binding to plasma macromolecules and/or reaction with small molecules in plasma such as reduced glutathione, cysteine, etc.

As mentioned above, the functional group of choice for the derivatization reaction would appear to be the terminal amino group. It was accordingly undertaken to compare three derivatization reactions specific for primary amines, and this work is presently in progress. The three reactions are:

1. Sanger reaction, reaction of 1-fluoro-2,4-dinitrobenzene (FDNB) with amino groups in alkaline solution, to produce a dinitrophenyl derivative of the amine. The DNP-amine is yellow and can be detected by spectrophotometry. This reaction is still under consideration, but some of the disadvantages for this application include: requires some length of time (from 15 minutes to 2 hours); reacts with thiols as well as amines so that it could serve to shift the equilibrium between WR 2721 and its metabolites; reaction does not go to completion so spiking of samples would have to be carried out to obtain estimate of yield for each analysis; a large excess of reagent is required which must then be extracted in the clean-up procedures.

2. Fluorescamine reaction, in which the nonfluorescent fluorescamine reacts with primary amines to produce a fluorescent product which is water-soluble and quite stable.

3. Reaction with o-phthalaldehyde and mercaptoethanol to form a fluorescent derivative. An analytical procedure for the quantification of WR 2721 for use in the in vitro studies was reported in Section III.C.1. This procedure will be compared with the fluorescamine reaction; one possible drawback for the present application is that it may not be as specific as fluorescamine for amino groups, but is reported to react as well with other biological compounds such as glutathione, arginine, agmatine, and 5-hydroxy- and 5-methoxy-indole compounds in addition to the free amino acids and other primary amines.

The derivatives of WR 2721, and its mercaptan (RSH) and disulfide (RSSR) metabolite forms produced by reactions (1), (2), and (3) above are being compared as to ease of formation, stability, linearity of response, detection limits, and other considerations such as stability of reagents, cost, ease of reaction, etc.

c. Results

(1) Derivatization with 1-Fluoro-2,4-dinitrobenzene

Figure 3 shows the linearity of absorbance of the DNP-derivatives of WR 2721 and the free mercaptan as a function of increasing volume of reaction product. Because neither the yield of this particular reaction nor the molar absorbance coefficient of the DNP-WR 2721 are known, it was not possible to correlate concentration of derivative with absorbance. The response appears to be linear only over a narrow concentration range.

(2) Derivatization with Fluorescamine

Results of initial experiments in which WR 2721 was derivatized with fluorescamine (4-phenylspiro[furan-2(3H),1'-phthalan]-3,3'-dione) appeared so promising that experimental effort has been concentrated on the study of this reaction. Fluorescamine itself is nonfluorescent but it reacts easily, rapidly, and directly with primary amines to form fluorophors (amine-fluorescamine adducts) which are stable for several hours. Neither excess fluorescamine nor its hydrolysis products are fluorescent. The derivatization reaction proceeds almost to completion (80 to 95%) within a fraction of a second at room temperature even if fluorescamine is not present in large excess. The extent of reaction which takes place is markedly influenced by pH; the optimal pH varies with the class of molecule to which the amino group is bonded, but is generally within the pH range of 8 to 9.5.[3]

For derivatization of WR 2721 with fluorescamine, reaction conditions used were similar to those described in the literature.[3,4] Reaction conditions were: 0.50 mL buffer, 0.20 mL drug solution + water; 0.50 mL fluorescamine solution (25 mg/100 mL acetone) added while vortexing. After vortexing, 0.50 mL sample was withdrawn for measurement of fluorescence; fluorescence was measured at 475 nm with excitation at 396 nm (excitation slit 6 nm and emission slit 10 nm) on an Hitachi-Perkin Elmer MPF-2A spectrofluorometer. The pH of the assay buffer was varied as well as the volume and concentration of drug solution; the volume of drug solution was varied from 0.010 to 0.100 mL with water added in the amount of 0.200 mL--vol (mL) drug solution. To vary the concentration of drug solution added, 1 mg WR 2721, its mercaptan, or disulfide was dissolved in varying volumes of buffer, pH 8.5 to 9.2. The pH of this sample buffer was higher than the assay buffer in order to maximize stability of WR 2721 in solution, but the amount added to the assay mixture was generally not large enough to appreciably affect the pH of the assay mixture.

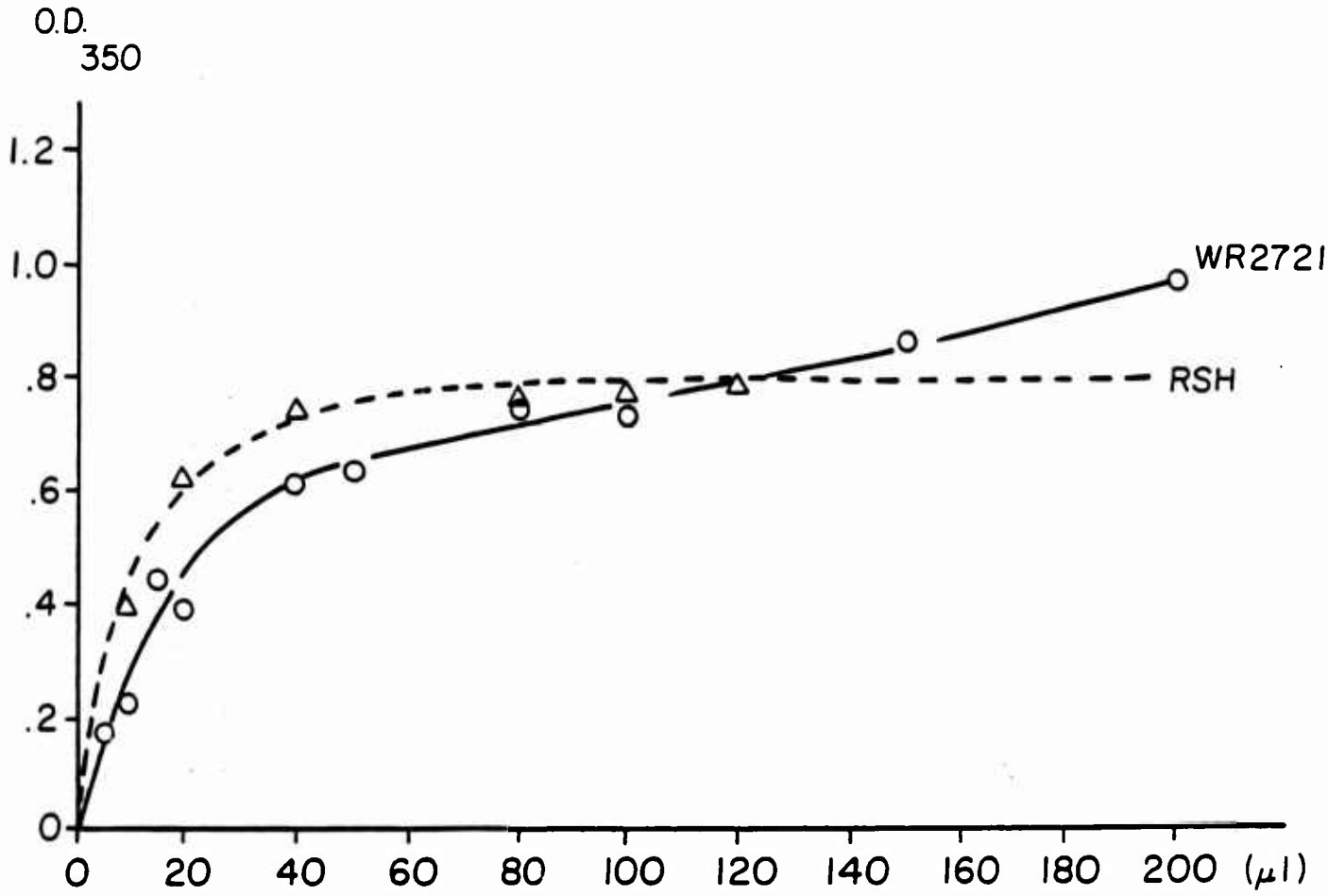


Figure 3. Absorbance of DNP-Derivatives of WR 2721 and RSH as Function of Concentration

In order to determine the optimum pH for the derivatization with fluorescamine, a series of buffers with pH values varying by 0.2 pH units was prepared by adjusting the pH of a 0.15 M solution of boric acid with concentrated ammonium hydroxide; these buffers were then used as assay buffers for the derivatization reaction while holding the concentration of WR 2721 and all other conditions constant. The pH profile shown in Figure 4 was obtained which suggests that a pH value of 7.6 to 8.0 would produce maximal fluorescence from the reaction of fluorescamine with WR 2721. Since WR 2721 appears to be slightly more stable with respect to hydrolytic cleavage of the phosphate group at pH 8.0 than pH 7.0 (Section IV.B), a pH of 7.8 was selected for the derivatization reaction. Unless specified, all reactions have been carried out in this borate buffer at pH 7.8.

To evaluate the linearity of fluorescence emission with concentration of WR 2721, its mercaptan, and disulfide, the influence of concentration upon fluorescence was measured for all three compounds and is shown in Figure 5. Although the emission continues to increase in a linear fashion as the concentration of drug increases up to close to a 1:1 stoichiometric ratio of drug to fluorescamine, the slope of the line obtained changes abruptly becoming flatter at high concentrations, particularly for the disulfide compound (Figure 5c). The biphasic response is less marked for the mercaptan and not clear-cut for the parent compound, WR 2721. The flattening of the emission response is probably due to self-quenching, i.e., bimolecular quenching, as the concentration of derivatized molecules in solution increases. The fact that the change of slope occurs at a slightly lower concentration (by weight) for the disulfide as compared to the mercaptan, and that the slope of the high-concentration line is flatter for the disulfide, suggests that intramolecular quenching as well as intermolecular quenching may be involved; this effect might be expected since both ends of the difulfide molecule would contain a fluorophor whereas only one end of the mercaptan molecule would. If the difference in slope is reproducible, it might be possible to exploit differences in quenching to estimate the mercaptan-disulfide content.

In order to determine whether the observed quenching was influenced by reaction conditions, the effects of both buffer composition and pH were explored in a preliminary fashion. Although a borate buffer is usually employed for the fluorescamine derivatization, phosphate buffer can also be used.[4] When the pH was held constant at 7.8 and borate buffer compared with an ammonium phosphate-$NH_4OH$ buffer, the same biphasic response was observed for the borate buffer with an inflection point at 4 to 5 µg/mL WR 2721; the ammonium phosphate buffer, however, gave a linear response with no change in slope, the slope being the same as observed for the higher concentration range in borate buffer. A triethylamine-phosphate buffer[5] was also tried, but the triethylamine on hand contained fluorescent impurities; this system will be evaluated in the future.

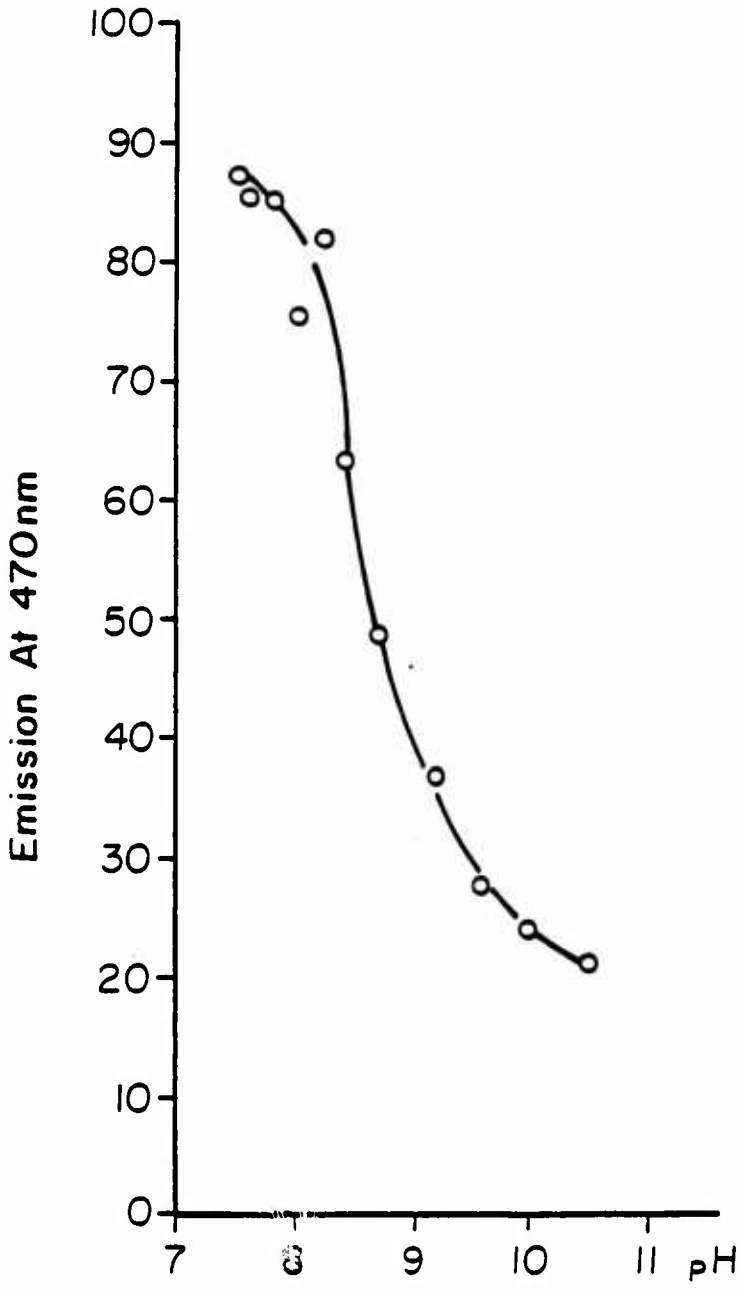


Figure 4. Fluorescence of WR 2721 Fluorescamine Adduct as Function of pH

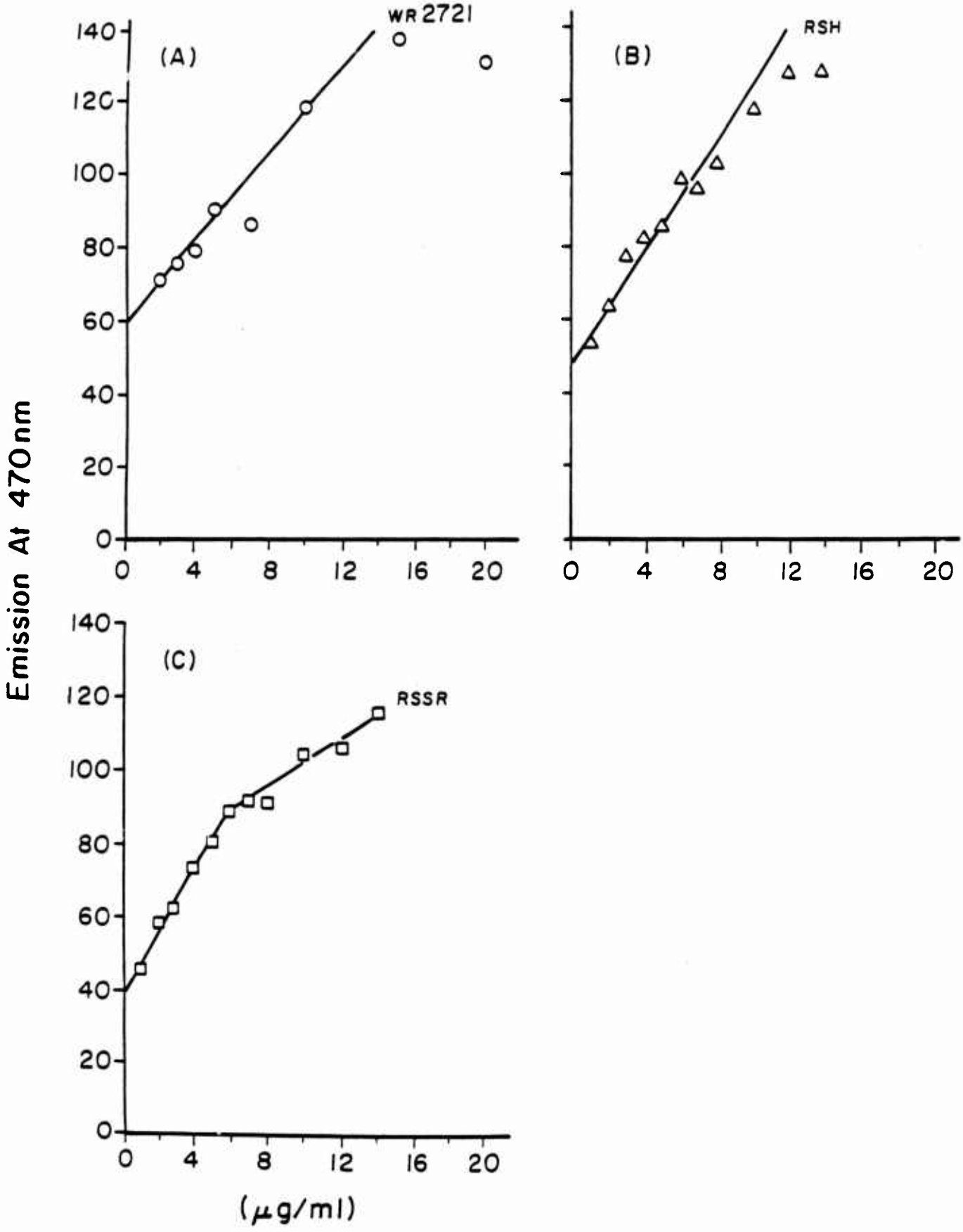


Figure 5. Emission at 470nm (arbitrary units) of fluorescamine adducts of WR 2721, RSH, and RSSR as function of concentration (μg/ml) at pH 7.8 excitation was at 399 nm. (A) WR 2721, (B) RSH, (C) RSSR.

Using only the borate buffer, the effect of pH upon linearity of response was explored (Figure 6). In Figure 6A the typical change in slope occurring at 6 µg/mL for pH 7.8 was obtained; when the pH was raised to 8.2, a biphasic response was also obtained but the inflection point increased to 8 µg/mL (Figure 6B); a pH of 8.7 shifted the inflection point to 14 µg/mL (Figure 6C); while a pH of 9.2 gave a straight line over the concentration range of 0 to 20 µg/mL (Figure 6D). In addition to this pH-associated shift in the inflection point, another feature of the plots shown in Figure 6, which changes in a consistent manner as pH is varied, is the slope of the low-concentration line - i.e. as the pH is increased, not only is the fluorescence emission obtained from a given concentration of WR 2721 decreased, but the rate at which the fluorescence increases as the drug concentration increases is also affected. All of these features - the shift in inflection point, the depression of fluorescence, and the decrease in slope - which are assocated with increasing pH are probably directly related to the pH profile of fluorescence (Figure 4) which exhibits a rather sharp pH range for optimal fluorescence. For amines in general this rather specific pH requirement is attributed to a competition between amine protonation and fluorescamine hydrolysis; if the pH is too low, the amine will be protonated limiting its availability for reaction, while if the pH is too high reagent hydrolysis predominates.[6] These competing processes determine the extent of the fluorogenic reaction - hence the fluorescence intensity yield per mole amine. For the reactions between fluorescamine and WR 2721 shown in Figure 6, the pH values employed were below the range at which fluorescamine hydrolysis becomes controlling, therefore the equilibria between protonated and unprotonated forms of amine should theoretically be the controlling factor. If this is true, the fluorescence should increase as the pH is raised since the extent of amine protonation would be expected to be decreasing. However, just the opposite effect appears to occur. If, in fact, the $pK_a$ of the primary amine nitrogen of WR 2721 is between pH 7-8, then deprotonation appears to be associated with some effect which limits the availability of this group for participation in the formation of fluorophor.

The fact that a sharp departure from linearity (shown as an abrupt change in slope in Figure 6) occurs is evidence of inner-filter quenching resulting from high fluorophor concentration.[6] It has been reported that concentration-dependent non-linearity can be improved by shifting the excitation wavelength from 390 nm to 340 nm and this change will be evaluated. However, for the purposes of this assay, potential problems due to non-linearity can probably be avoided by specifying reaction conditions with respect to ratio of fluorescamine reagent to amine, suitable dilutions of amine, pH of assay, etc.

The apparent quenching of fluorescence observed above 6 µg/mL WR 2721 at pH 7.8, and the fact that these measurements were obtained at fairly low gain or spectrofluorometer sensitivity settings, suggested that lower concentrations of drug should be explored. To assess the detectability of this assay method, the plots shown in Figure 7 were obtained. In these experiments, the lowest concentration which could be reliably measured was 0.08 µg/mL WR 2721 or 0.37 nmoles/mL. Below this

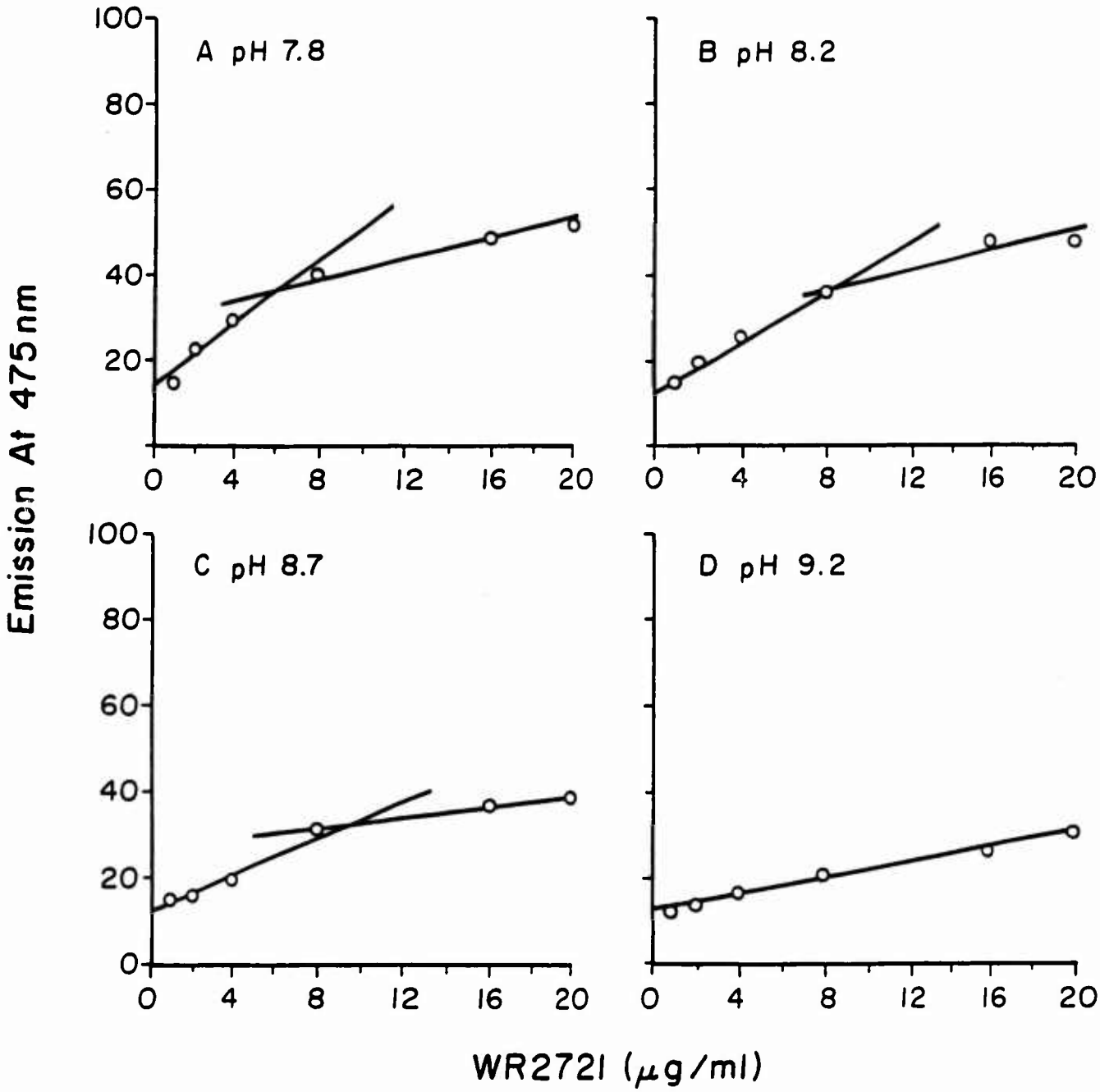


Figure 6. Linearity of fluorescence intensity yield as function of pH of fluorescamine-WR 2721 reaction. (A) pH 7.8; (B) pH 8.2; (C) pH 8.7; (D) pH 9.2; 0.15 M boric acid - $NH_4OH$ buffer.

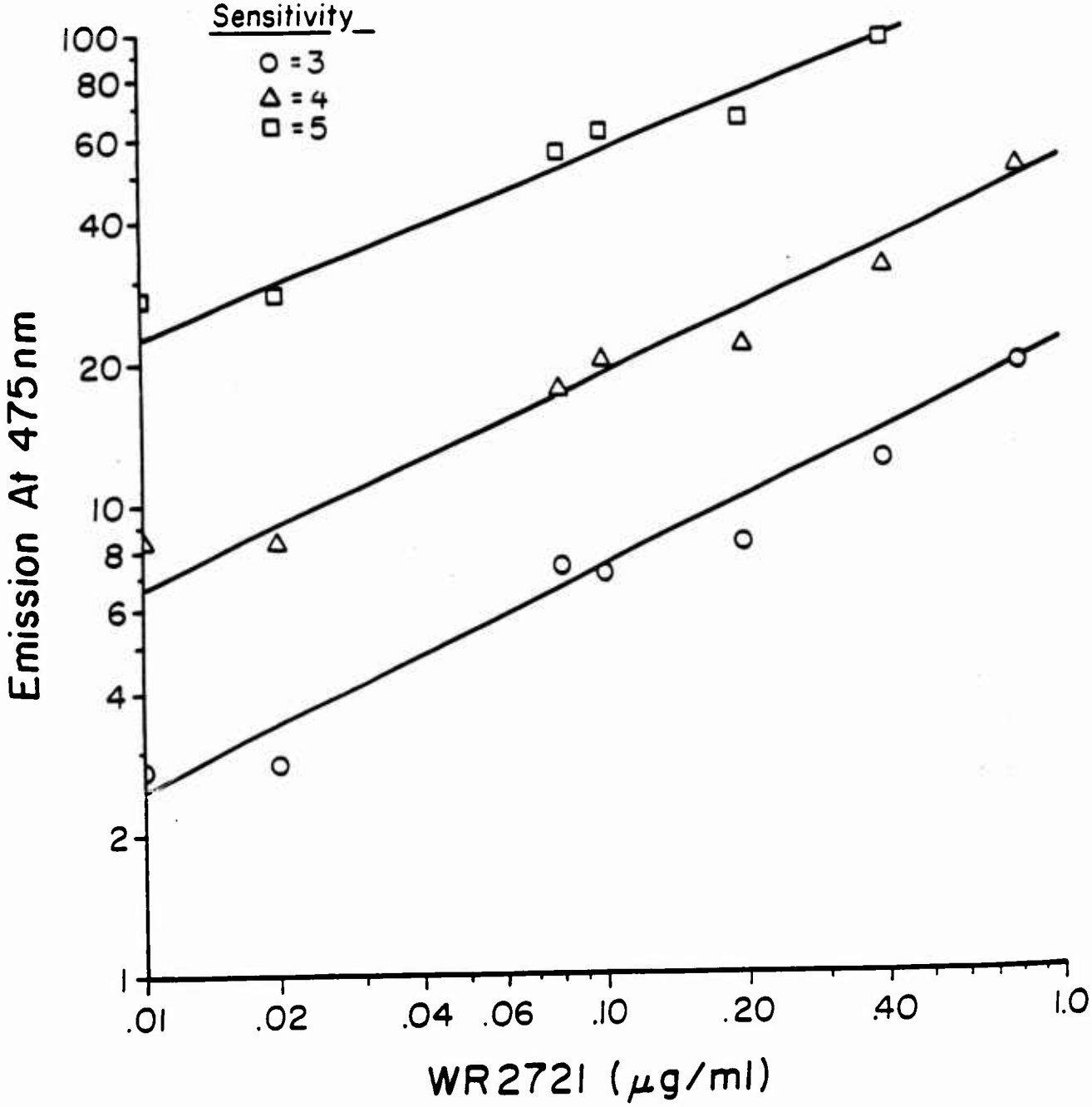


Figure 7a. Fluorescence emission of fluroescamine-WR 2721 derivative obtained at various spectrofluorometer sensitivity settings as function of WR 2721 concentration (0-01 - 1.0 micrograms/mL).

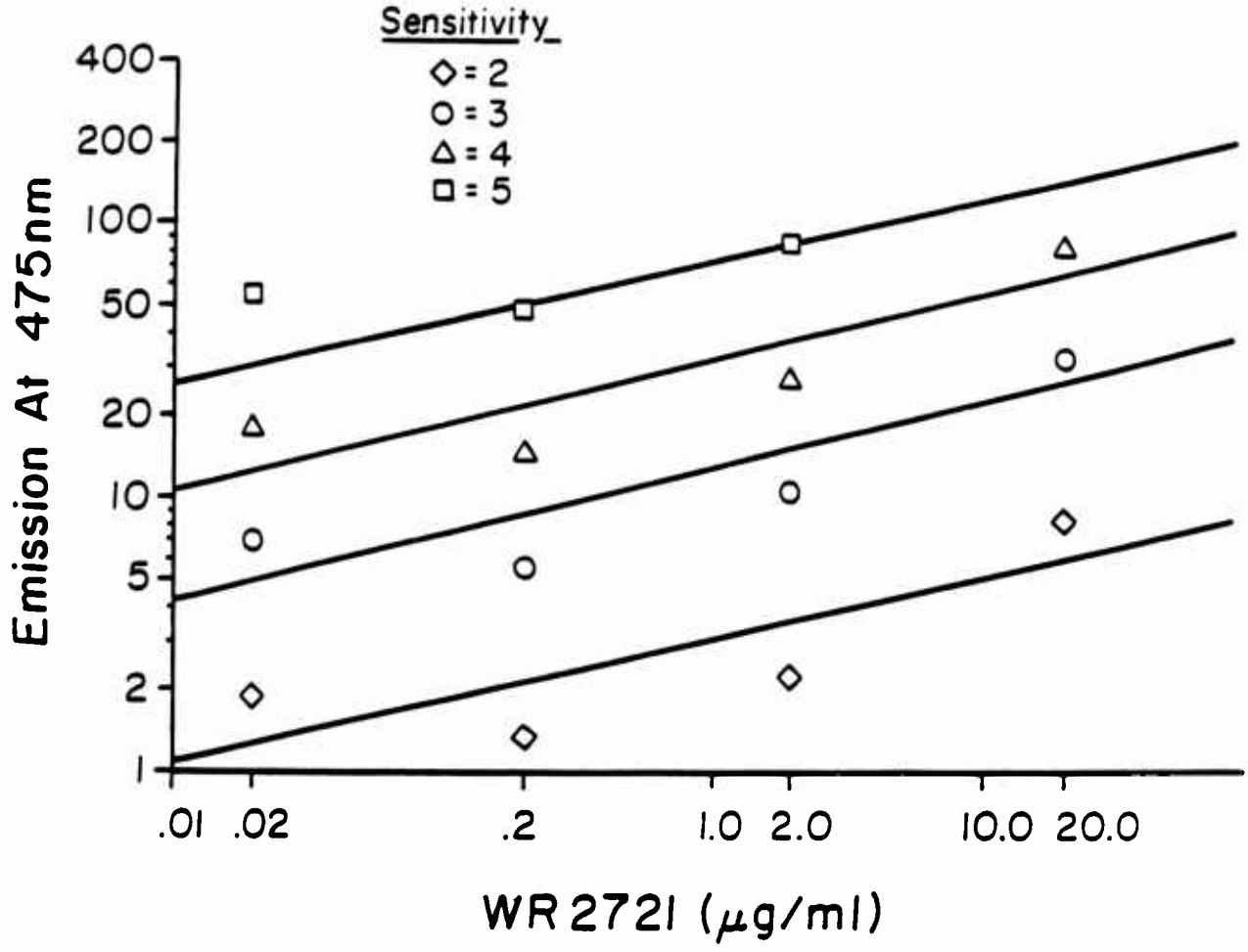


Figure 7b. Fluorescence emission of fluorescamine-WR 2721 derivative obtained at various spectrofluorometer sensitivity settings as function of WR 2721 concentration (0.01 - 20.0 micrograms/mL).

concentration, fluorescent contributions from reaction mixture components became overriding; however, sensitivity limits of the instrument have certainly not been reached, and the limits of the derivatization reaction also do not appear to have been attained. Limitation at this point appears to be due to contamination of buffers with fluorescent impurities. Acetone was the only spectroquality chemical employed in these experiments; efforts to obtain entirely fluorescence-free reagents are presently underway and the effects of these reagents upon the limits of detectability will be measured.

It is anticipated that this assay will be tailored to HPLC instrumentation and techniques. Fluorescamine has been employed for HPLC derivatization in both pre- and post-column systems employing silica gel and reversed-phase columns.[7,8,9] In the preliminary studies to date, thin layer chromatography (TLC) systems have been employed to explore chromatographic characteristics of the fluorescamine derivatives of WR 2721, its mercaptan and disulfide. On reversed-phase TLC plates with acetonitrile-water (20:80) as developing solvent, the fluorescamine derivative of WR 2721 moves close to the solvent front while the other two derivatives appear to barely move from the origin and are not well separated. Increasing the acetonitrile concentration to 50% apparently reverses the order of relative mobilities with an $R_f$ for WR 2721 of .70 and .82 for the RSH form. A more hydrophobic solvent system, isopropanol-$CHCl_3$-$NH_4OH$-$H_2O$ (70:30;18:7) on silica gel TLC[10] produces fairly good resolution of the three species; relative mobilities of .49 (WR 2721); .57 (mercaptan); and .63 (disulfide) were obtained. In both acetonitrile-$H_2O$ systems, there appeared to be no detectable conversion of WR 2721 to mercaptan or disulfide forms during TLC analysis, but there did appear to be conversion either of derivatized mercaptan to derivatized disulfide or vice versa in that the same spot was obtained for both reduced and oxidized compounds. The more hydrophobic solvent system (isopropanol-$CHCl_3$-$NH_4OH$-$H_2O$) did resolve these two compounds - perhaps by limiting autooxidation (or reduction) conversions which may occur more readily in aqueous systems. Derivatization with fluorescamine obviously makes all three compounds more readily soluble in organic solvents and the differences in polarity between WR 2721 and the mercaptan/disulfide species after derivatization due to the presence of the polar phosphate group on WR 2721 provides good resolution of drug and metabolite forms. The possibility of derivatization with fluorescamine in plasma and extraction of derivatives into lipophilic solvents followed by pre-column cleanup of extracts is being investigated.

# IV. RESULTS

## A. Microencapsulation

Process operating conditions were initially established by several encapsulation runs in which placebo microspheres were prepared in the desired size range. Conditions for these runs are presented in Table III. No WR 2721 was used in these runs in order to conserve the drug. Parameters varied were system temperature, head speed, nozzle size, and feed rate in order to delineate the conditions necessary to produce acceptable microspheres of various sizes from 250 to 841 microns. Ambient temperature was held constant at 22.2°C. Microspheres <707 microns were partially flattened due to insufficient cooling during production. Therefore, to obtain satisfactory capsules <707 microns, it was necessary to raise the head of the centrifugal encapsulation device to a height greater than five feet to allow additional cooling and hardening of the microspheres prior to contact with the collection surface. WR 2721-containing microspheres and microcapsules using matrices composed of glycerides, stearic acid, and paraffin wax were then successfully prepared. Details of the encapsulation runs are presented in Tables IV and V.

## B. Hydrolytic Stability of Encapsulated WR 2721

Hydrolytic stability tests at pH 1 were carried out on samples from 51 experimental encapsulation runs. A "protection factor" has been defined as the fraction of WR 2721 remaining in the formulation after testing relative to the fraction of unencapsulated WR 2721 remaining after being subjected to the same acidic conditions. This factor has been calculated for all formulations and the data are presented in Table VI. Eight runs produced samples which retained greater than 73 percent of the original payload at the conclusion of the test. Five of these (Runs 1-14B, 1-22, 1-11C, 1-10C and 1-27) were clearly superior, retaining greater than 85 percent of the drug. These five were also tested at pH 3, at the same temperature and for the same length of time. The results were similar, with 75-90 percent of the drug being retained (Table VII).

## C. In Vitro Release Rates

In vitro release rate studies have been initiated on the five formulations found to be most promising in the hydrolytic stability tests. The study is incomplete, but the partial results obtained to date may indicate the direction of future encapsulation formulation work.

Seven rate tests were completed with all samples releasing 95 percent of their payloads within 94 minutes. The fastest five release rates were obtained on microsphere formulations, 95 percent release occurring within 12-30 minutes. Slowest times for 95 percent release were obtained on microcapsule formulations, the values being 48 and 94 minutes. Microspheres with matrices of stearic acid/paraffin wax and of stearic acid/triglyceride gave similar release rates. These results are summarized in Table VIII.

TABLE III. PROCESS CONDITIONS

| Run No. | Matrix Material | System Temp. (°C) | Head Speed (RPM) | Nozzle Size (inches) | Feed Rate (g/m) | Size (μ) Distribution (%) <250 | 250-500 | 500-707 | 707-841 | Comment |
|---|---|---|---|---|---|---|---|---|---|---|
| 1-3A | Emersol 6349 | 65-68 | 1400 | 0.020 | 24 | 1 | 31 | 68 | - | Product all spherical. Satisfactory run conditions. |
| 1-3B | Emersol 6349 | 65-68 | 1600 | 0.020 | 24 | 2 | 69 | 29 | - | Product all spherical. Satisfactory run conditions. |
| 1-3C | Atmul 84K | 68-71 | 1500 | 0.020 | 30 | | 35 | 65 | - | >707 μ partially flattened on impact. Head height too low (4'6" above base). |
| 1-3D | Atmul 84K | 65-68 | 1000 | 0.031 | 22 | | 43 | 57 | | >707 μ partially flattened on impact. Head height too low (4'6" above base). |
| 1-3E | Emersol 6349 | 65-68 | 900 | 0.020 | 27 | | 65 | 35 | | >707 μ partially flattened on impact. Head height too low (4'6" above base). |

TABLE IV. WR 2721 MICROSPHERE PRODUCTION[a,b,c,d]

| Run No. | Matrix Composition (%)[e] WR 2721 | Grocol 600-E | Grocol 55-E | 60N HTG | Atmul 84K | Emersol 6349 | Emersol 6351 | Alfol 16 NF | Alfol 18USP | Emery 6354 | Paraffin | Temp. of System (°C) | Anal. % WR 2721 | Size (μ) Distribution (%) 250-500 | 500-707 | 707-841 | 841-1000 | <250+ >1000 | Comments |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1-4 | 25.0 | | | | | 75.0 | | | | | | 65.5-68.3 | | 3.9 | 70.9 | 24.6 | 0.6 | | Satisfactory appearing product. Run conditions: head speed (RPM) 1400. |
| 1-5 | 20.0 | | | | 80.0 | | | | | | | " | | 4.6 | 48.6 | 41.8 | 5.0 | | >841 μ have flat side. Run conditions: head speed (RPM) 1400. |
| 1-6A | 20.0 | | | 80.0 | | | | | | | | " | | 15.2 | 71.4 | 0.4 | 10.8 | 2.2 | >707 μ have flat side. Run conditions: head speed (RPM) 1400; feed rate (g/m) 28. |
| 1-6B | 25.0 | | | | | 75.0 | | | | | | " | | 7.9 | 57.3 | 34.5 | 0.3 | | Satisfactory appearing product. Run conditions: head speed (RPM) 1500; feed rate (g/m) 29. |
| 1-6C | 20.0 | | | 80.0 | | | | | | | | " | | 21.1 | 66.7 | 10.0 | 2.2 | | >707 μ have flat side. Run conditions: head speed (RPM) 1600; feed rate (g/m) 30. |
| 1-7A | 20.0 | | | | | | | 80.0 | | | | 52.0-57.0 | 34.8 | 16.0 | 54.6 | 24.4 | 2.1 | 2.9 | Unsatisfactory product (plugged nozzle). |
| 1-7B | 20.0 | | | | | | | | 80.0 | | | " | 38.0 | 17.0 | 69.0 | 13.1 | 0.9 | | Product appears to be satisfactory. Run conditions: head speed (RPM) 1750; feed rate (g/m) 20. |
| 1-7C | 20.0 | | | | | | | | | 20.0 | | " | 38.7 | 49.5 | 48.4 | 0.5 | | 1.6 | Same as Run 1-7B. |
| 1-8A | 20.0 | | | | 40.0 | 40.0 | | | | | | 62.0-66.0 | 20.8 | 20.4 | 74.1 | 3.8 | 0.9 | 0.8 | Product appears to be satisfactory. Run conditions: head speed (RPM) 1750; feed rate (g/m) 20. |
| 1-8B | 20.0 | | | 40.0 | 40.0 | | | | | | | " | 28.6 | 26.0 | 51.9 | 15.6 | | 6.5 | Same as Run 1-8A. |
| 1-9A | 20.0 | 80.0 | | | | | | | | | | 65.5-68.3 | 19.1 | 16.7 | 83.3 | | | | >707 μ were lost on collection due to insufficient cooling. |
| 1-9B | 20.0 | | | | | | 80.0 | | | | | 71.1-73.9 | 16.0 | 15.3 | 80.1 | 4.6 | | | Satisfactory appearing product. |

| Run No. | Matrix Composition (%)[e] WR 2721 | Grocol 600-E | Grocol 55-E | 60N HTG | Atmul 84K | Emersol 6349 | Emersol 6351 | Alfol 16 NF | Alfol 18USP | Emery 6354 | Paraffin | Temp. of System (°C) | Anal. % WR 2721 | Size (μ) Distribution (%) 250-500 | 500-707 | 707-841 | 841-1000 | <250+ >1000 | Comments |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1-9C | 20.0 | | 80.0 | | | | | | | | | 65.5-68.3 | 19.5 | 11.5 | 80.3 | 8.2 | | | satisfactory appearing product. |
| 1-10A | 20.0 | 70.0 | | | | | | | | | 10.0[j] | ~68.3 | 18.5 | 21.5 | 78.5 | | | | >707 μ were lost on collection due to insufficient cooling. |
| 1-10B | 20.0 | | | 70.0 | | | | | | | 10.0[j] | | 20.5 | 20.7 | 79.3 | | | | >707 μ were lost on collection due to insufficient cooling. |
| 1-10C | 20.0 | | | | | 70.0 | | | | | 10.0[j] | | 19.5 | 42.7 | 57.3 | | | | >707 μ were lost on collection due to insufficient cooling. |
| 1-10D | 20.0 | 35.0 | | | | 35.0 | | | | | 10.0[j] | | 18.8 | 45.8 | 54.2 | | | | >707 μ were lost on collection due to insufficient cooling. |
| 1-11A | 20.0 | | | | | | 70.0 | | | | 10.0[j] | ~73.9 | 18.4 | 28.1 | 68.9 | 3.0 | | | Satisfactory appearing product. |
| 1-11B | 20.0 | | 70.0 | | | | | | | | 10.0[j] | ~68.3 | 19.7 | 33.0 | 65.2 | 1.8 | | | Satisfactory appearing product. |
| 1-11C | 20.0 | 40.0 | | | | 40.0 | | | | | | | 18.9 | 39.5 | 60.5 | | | | Satisfactory appearing product. |
| 1-19 | 20.0 | | | | | 70.0 | | | | | 10.0[j] | " | 17.6 | 55.8 | 44.2 | | | | Made well. Run conditions: head speed (RPM) 2450; feed rate (g/m) 20; room temperature 16.7°C (62°F). |
| 1-20 | 20.0 | 40.0 | | | | 40.0 | | | | | | " | 16.5 | 48.8 | 51.2 | | | | Made well. Run conditions: head speed (RPM) 2200; feed rate (g/m) 20; room temperature 16.7°C (62°F). |
| 1-24[f] | 20.0 | | | | | 70.0 | | | | | 10.0 | " | 17.7 | 20.0 | 80.0 | | | | Made well. Run conditions: head speed (RPM) 1950; feed rate (g/m) 22; room temperature 16.7°C (62°F). |
| 1-25[g] | 20.0 | 40.0 | | | | 40.0 | | | | | | " | 16.8 | 15.2 | 84.8 | | | | Made well. Did have some start-up problems. Run conditions: RT rose to 20.3°C (68.5°F) during run; head speed (RPM) 1950; feed rate (g/m) 22. |

| Run No. | Matrix Composition (%)[e] | | | | | | | | | | Temp. of System (°C) | Anal. % WR 2721 | Size (μ) Distribution (%) | | | | Comments |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | WR 2721 | Grocol 600-E | Grocol 55-E | 60N HTG | Atmul 84K | Emersol 6349 | Emersol 6351 | Alfol 16 NF | Alfol 18USP | Emery 6354 | Paraffin | | | 250-500 | 500-707 | 707-841 | 841-1000 | <250+ >1000 | |
| 1-27[f] | 20.0 | | | | | 70.0 | | | | | 10.0[j] | " | 19.1 | 16.2 | 76.2 | 7.6 | | | Made well. Run conditions: head speed (RPM) 1850; feed rate (g/m) 23; room temperature 16.7°C (62°F). |
| 1-28[g] | 20.0 | 40.0 | | | | 40.0 | | | | | | " | 18.9 | 13.2 | 80.2 | 6.6 | | | Made well. Run conditions: head speed (RPM) 1900; feed rate (g/m) 23-24; room temperature 16.7°C (62°F). |
| 1-32 | 40.0 | | | | | 52.5 | | | | | 7.5[k] | " | - | - | - | | | | No capsules produced due to WR 2721 settling out of dispersion. Run conditions: head speed (RPM) 1780; feed rate (g/m) 23; room temperature 17.8°C (64°F). |
| 1-33 | 30.0 | | | | | 61.2 | | | | | 8.8[k] | " | 25.3 | 29.6 | 70.4 | | | | Made capsules. Had plugging during run. Poor capsules. Run conditions: head speed (RPM) 1780; feed rate (g/m) 23; room temperature 17.8°C (64°F). |
| 1-34[h] | 40.0 | | | | | 52.5 | | | | | 7.5[k] | " | 14.2 | 40.0 | 60.0 | | | | Made capsules. Had large loss on collection. Molten solution containing WR 2721 was slightly thixotropic. Had some plugging during run. Poor capsules. Run conditions: head speed (RPM) 1780; feed rate (g/m 23; room temperature 17.8°C (64°F). |
| 1-35[h] | 35.0 | | | | | 56.9 | | | | | 8.1[k] | " | 30.8 | 44.9 | 55.1 | | | | Made capsules. Had plugging during run. Poor capsules. Run conditions: head speed (RPM) 1780; feed rate (g/m) 15; room temperature 17.8°C (64°F). |
| 1-36A[h] | 35.0 | | | | | 56.9 | | | | | 8.1[k] | " | 29.4 | 25.6 | 74.4 | | | | Made well. Some capsules were lost on collection. Poor capsules. Room temperature 19.4°C (67°F). Nozzle size used 0.033 inches. |

| Run No. | Matrix Composition (%)[e] WR 2721 | Grocol 600-E | Grocol 55-E | 60N HFG | Atmul 84 | Emersol 6349 | Emersol 6351 | Alfol 16 NF | Alfol 18USP | Emery 6354 | Paraffin | Temp. of System (°C) | Anal. % WR 2721 | Size (μ) Distribution (%) 250-500 | 500-707 | 707-841 | 841-1000 | <250+ >1000 | Comments |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1-36B[h] | 35.0 | 32.5 | | | | 32.5 | | | | | | " | 33.6 | 34.4 | 65.6 | | | | Made well. Some capsules were lost on collection. Room temperature 19.4°C (67°F). Nozzle size used 0.033 inches. |
| 1-37A[h] | 40.0 | | | | | 52.5 | | | | | 7.5[k] | " | 33.5 | 25.0 | 75.0 | | | | Made well. Some capsules were lost on collection. Poor capsules. Room temperature 17.8°C (64°F). Nozzle size used 0.033 inches. |
| 1-37B | 40.0 | 30.0 | | | | 30.0 | | | | | | " | 38.5 | 27.8 | 72.2 | | | | Made well. Some capsules were lost on collection. Poor capsules. Room temperature 17.8°C (64°F). Nozzle size used 0.033 inches. |
| 1-38A[h] | 45.0 | | | | | 48.1 | | | | | 6.9[k] | " | 39.3 | 45.2 | 54.8 | | | | Made well. Some capsules were lost on collection. Poor capsules. Room temperature 19.4°C (67°F). Nozzle size used 0.033 inches. |
| 1-38B[h] | 45.0 | 27.5 | | | | 27.5 | | | | | | " | 36.9 | 34.2 | 65.8 | | | | Made well. More capsules were lost on collection compared to Run 1-38A. Poor capsules. Room temperature 19.4°C (67°F). Nozzle size used 0.033 inches. |
| 1-40A[h] | 30.0 | 35.0 | | | | 35.0 | | | | | | 68.3-71.1 | 27.6 | 15.7 | 84.3 | | | | Made capsules. Poor capsules. Run conditions: feed rate (g/m) 22; room temperature 16.7°C (62°F). |
| 1-40B[h] | 30.0 | | | | | 61.2 | | | | | 8.8[k] | " | 28.8 | 36.1 | 63.9 | | | | Made capsules well. Run conditions: feed rate (g/m) 22; room temperature 16.7°C (62°F). |
| 1-40C[h] | 30.0 | 35.0 | | | | 35.0 | | | | | | " | 29.9 | 22.3 | 77.7 | | | | Made capsules. Poor capsules. Run conditions: feed rate (g/m) 22; room temperature 16.7°C (62°F). |
| 1-41[i] | 34.8 | 32.6 | | | | 32.6 | | | | | | " | | 16.1 | 83.9 | | | | Made capsules. Run conditions: feed rate (g/m) 22; room temperature 16.7°C (62°F). |

| Run No. | Matrix Composition (%)[e] WR 2721 | Grocol 600-E | Grocol 55-E | 60N HTG | Atmul 84K | Emersol 6349 | Emersol 6351 | Alfol 16 NF | Alfol 18USP | Emery 6354 | Paraffin | Temp. of System (°C) | Anal. % WR 2721 | Size (μ) Distribution (%) 250-500 | 500-707 | 707-841 | 841-1000 | <250+ >1000 | Comments |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1-42[i] | 34.6 | | | | | 57.4 | | | | | 8.0[k] | " | | 19.5 | 80.5 | | | | Made capsules. Run conditions: feed rate (g/m) 22; room temperature 16.7°C (62°F). |

a. Nozzle size used: 0.020 inches (inside only) unless noted under comments.
b. Feed rate (g/m) for all runs: 24 unless noted under comments.
c. Head speed (RPM) for all runs: 1720 unless noted under comments.
d. Room temperature: 22.2°C (72°F) unless noted under comments.
e. WR 2721 dispersed in molten material. Mixture kept under nitrogen blanket.
f. Attempted repeat of Run 1-10C.
g. Attempted repeat of Run 1-11C.
h. WR 2721 particle size was reduced (utilizing a mortar and pestle).
i. WR 2721 particle size was reduced (utilizing a mortar and pestle) and WR 2721 particle size used was less than 105 microns (utilizing a sieve).
j. Paraffin N.F.
k. Fully refined Paraffin Wax 140/145. (Production Paraffin Wax N.F. discontinued).

TABLE V. WR 2721 MICROCAPSULE PRODUCTION[a,b,c]

| Run No. | Shell Composition (%) | Fill Composition (%) | Feed Rate (g/min) Shell | Feed Rate (g/min) Fill | System (°C) | Theoret. % WR 2721 | Analysis % WR 2721 | Size (μ) Distribution (%) 250-500 | 500-707 | 707-841 | Comment |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1-12A | Emersol 6349 | 80% Emersol 6349<br>20% WR 2721 | 9.0 | 18.0 | 68.3-71.1 | 13.3 | 12.9 | 22.4 | 77.6 | | Mech. encapsulation problems. |
| 1-12B | Grocol 55-E | 80% Grocol 55-E<br>20% WR 2721 | 9.0 | 22.0 | " | 14.2 | 11.9 | 16.0 | 74.0 | 10.0 | Satisfactory run. |
| 1-12C | Emersol 6349 | 80% Emersol 6349<br>20% WR 2721 | 9.0 | 22.0 | " | 14.2 | 12.5 | 15.9 | 77.2 | 6.9 | Repeat of Run 1-12A. Satisfactory run. |
| 1-13A | Emersol 6351 | 80% Emersol 6351<br>20% WR 2721 | 9.0 | 23.0 | 68.3-73.9 | 14.4 | 9.8 | 20.7 | 71.5 | 7.8 | Mech. encapsulation problems. |
| 1-13B | 60N HTG | 80% 60N HTG<br>20% WR 2721 | 9.0 | 23.0 | 68.3-71.1 | 14.4 | 13.3 | 27.8 | 72.2 | | >707 μ capsules were lost on collection due to insufficient cooling. Temperature may be too high, causing microcapsules to have a flat side. |
| 1-14A | Grocol 600-E | 80% Grocol 600-E<br>20% WR 2721 | 9.0 | 23.0 | " | 14.4 | 12.4 | 30.6 | 69.4 | | Same as Run 1-13B. |
| 1-14B | 50% Grocol 600-E<br>50% Emersol 6349 | 40% Grocol 600-E<br>40% Emersol 6349<br>20% WR 2721 | 9.0 | 23.0 | " | 14.4 | 16.0 | 72.8 | 27.2 | | Satisfactory appearing capsules. Shell seal problems--leaking. |
| 1-14C | 50% Grocol 600-E<br>50% Emersol 6349 | 40% Grocol 600-E<br>40% Emersol 6349<br>20% WR 2721 | 9.0 | 23.0 | " | 14.4 | 15.4 | 37.6 | 64.4 | | Repeat Run 1-14B. Mech. encapsulation problems. Plugging occurred during run. |
| 1-15 | 50% Grocol 600-E<br>50% Emersol 6351 | 40% Grocol 600-E<br>40% Emersol 6351<br>20% WR 2721 | 9.0 | 23.0 | " | 14.4 | 11.7 | 29.5 | 70.5 | | >707 μ capsules were lost on collection due to insufficient cooling. |
| 1-21 | 50% Grocol 600-E<br>50% Emersol 6349 | 40% Grocol 600-E<br>40% Emersol 6349<br>20% WR 2721 | 9.3 | 24.0 | " | 14.4 | 12.6 | 17.2 | 77.7 | 5.1 | Had some plugging during run. Condition: 15 mils spacing used. |
| 1-22[d] | " | " | 10.0 | 23.2 | " | 14.0 | 14.0 | 22.5 | 69.5 | 8.0 | Made well. Room temperature 16.7°C (62°F). |
| 1-26 | " | " | 10.1 | 25.0 | " | 14.3 | 13.8 | 6.8 | 78.8 | 14.4 | Made well. Had some capsules flatten on collection. Head speed could be too slow for the feed rates used (1800 RPM). RT rose from 16.7°C (62°F) to 18°C (64.5°F). |
| 1-39 | 50% Emersol 6349<br>50% Grocol 600-E | 27.5% Emer. 6349<br>27.5% Groc. 600-E<br>45.0% WR 2721 | 10.8 | 22.0 | " | 30.2 | 15.4 | 37.3 | 62.7 | | Made well. Did have some mechanical problems. Poor capsules. Nozzles used 0.033 inches inside, 0.046 inches outside, 10 mil spacing; head speed (RPM) 1980; room temperature 17.8°C (64°F). WR 2721 particle size was reduced (utilizing a mortar and pestle). |

a. Nozzles used: 0.020 inches inside, 0.040 inches outside, 12 mil spacing unless noted under comments.
b. WR 2721 dispersed in molten fill matrix. Mixture kept under nitrogen blanket.
c. Head speed (RPM) for all runs: 1750 unless noted under comments.
d. Attempted repeat of Run 1-14B.

TABLE VI. MICROSPHERE AND MICROCAPSULE ANALYSES AND HYDROLYSIS TEST RESULTS (pH 1)

| Run No.[a] | Theoretical[b] Payload % | Original[c] Assay % | After[d] Test Assay % | % WR 2721[e] Recovered | Protection Factor[f] pH 1.0 |
|---|---|---|---|---|---|
| 1-4[g] | 25.0 | 21.3 | 11.0 | 51.6 | 5.1 |
| 1-5[g] | 20.0 | 20.0 | 0.1 | 0.5 | 0.1 |
| 1-6A[g] | 20.0 | 19.5 | 11.1 | 56.9 | 5.6 |
| 1-6B[g] | 25.0 | 22.9 | 10.8 | 47.2 | 4.7 |
| 1-6C[g] | 20.0 | 19.4 | 12.9 | 66.5 | 6.6 |
| 1-7A[g] | 20.0 | 34.8 | 0.9 | 2.6 | 0.3 |
| 1-7B[g] | 20.0 | 38.0 | 0.9 | 2.4 | 0.2 |
| 1-7C[g] | 20.0 | 38.7 | 0.7 | 1.8 | 0.2 |
| 1-8A[g] | 20.0 | 20.8 | 1.8 | 8.7 | 0.9 |
| 1-8B[g] | 20.0 | 28.6 | 1.8 | 6.3 | 0.6 |
| 1-9A[g] | 20.0 | 19.1 | 2.0 | 10.5 | 1.0 |
| 1-9B[g] | 20.0 | 16.0 | 1.2 | 7.5 | 0.7 |
| 1-9C[g] | 20.0 | 19.5 | 0.4 | 2.1 | 0.2 |
| 1-10A[g] | 20.0 | 18.5 | 1.5 | 8.1 | 0.8 |
| 1-10B[g] | 20.0 | 20.5 | 13.2 | 64.4 | 6.4 |
| 1-10C[g] | 20.0 | 19.5 | 17.6 | 89.2 | 8.8 |
| 1-10D[g] | 20.0 | 18.8 | 7.6 | 40.4 | 4.0 |
| 1-11A[g] | 20.0 | 18.4 | 0.9 | 4.9 | 0.5 |
| 1-11B[g] | 20.0 | 19.7 | 3.8 | 19.3 | 1.9 |
| 1-11C[g] | 20.0 | 18.9 | 16.7 | 88.4 | 8.8 |
| 1-12A[h] | 13.3 | 12.9 | 3.7 | 28.7 | 2.8 |
| 1-12B[h] | 14.2 | 11.9 | 4.2 | 35.3 | 3.5 |
| 1-12C[h] | 14.2 | 12.5 | 7.0 | 56.0 | 5.5 |
| 1-13A[h] | 14.4 | 9.8 | 1.0 | 10.2 | 1.0 |
| 1-13B[h] | 14.4 | 13.6 | 1.8 | 13.2 | 1.3 |
| 1-14A[h] | 14.4 | 12.4 | 2.6 | 21.0 | 2.1 |
| 1-14B[h] | 14.4 | 16.0 | 15.1 | 94.4 | 9.3 |
| 1-14C[h] | 14.4 | 15.4 | 9.9 | 64.3 | 6.4 |
| 1-15[h] | 14.4 | 11.7 | 7.3 | 62.4 | 6.2 |
| 1-19[g] | 20.0 | 17.6 | 8.7 | 49.4 | 4.9 |
| 1-20[g] | 20.0 | 16.5 | 12.2 | 73.9 | 7.3 |
| 1-21[h] | 14.4 | 12.6 | 10.0 | 79.4 | 7.9 |
| 1-22[h] | 14.0 | 14.0 | 12.5 | 89.3 | 8.8 |
| 1-24[g] | 20.0 | 17.7 | 9.9 | 55.9 | 5.5 |
| 1-25[g] | 20.0 | 16.8 | 10.6 | 63.1 | 6.3 |
| 1-26[h] | 14.3 | 13.8 | 7.2 | 52.2 | 5.2 |
| 1-27[g] | 20.0 | 19.1 | 16.4 | 85.9 | 8.5 |
| 1-28[g] | 20.0 | 18.9 | 11.5 | 60.8 | 6.0 |

TABLE VI. (Cont'd)

| Run No.[a] | Theoretical[b] Payload % | Original[c] Assay % | After[d] Test Assay % | % WR 2721[e] Recovered | Protection Factor[f] pH 1 |
|---|---|---|---|---|---|
| 1-33[g] | 30.0 | 25.3 | 1.2 | 4.7 | 0.5 |
| 1-34[g] | 40.0 | 14.2 | 2.9 | 20.4 | 2.0 |
| 1-35[g] | 35.0 | 30.8 | 9.1 | 29.6 | 2.9 |
| 1-36A[g] | 35.0 | 29.4 | 9.3 | 31.6 | 3.1 |
| 1-36B[g] | 35.0 | 33.6 | 20.5 | 61.0 | 6.0 |
| 1-37A[g] | 40.0 | 33.5 | 9.4 | 28.1 | 2.8 |
| 1-37B[g] | 40.0 | 38.5 | 14.1 | 36.6 | 3.6 |
| 1-38A[g] | 45.0 | 39.3 | 6.1 | 15.4 | 1.5 |
| 1-38B[g] | 45.0 | 36.9 | 2.4 | 6.5 | 0.6 |
| 1-39[h] | 30.2 | 15.4 | 0.1 | 0.7 | 0.1 |
| 1-40A[g] | 30.0 | 27.6 | 9.4 | 34.1 | 3.4 |
| 1-40B[g] | 30.0 | 28.8 | 22.4 | 77.7 | 7.7 |
| 1-40C[g] | 30.0 | 29.9 | 15.3 | 51.2 | 5.1 |

a. Composition given in Tables IV and V.
b. Gravimetric composition of melt prior to microsphere or microcapsule production.
c. Determined by HPLC analysis, average of two determinations. An average of three determinations was used for Runs 1-4 through 1-8B.
d. Determined by HPLC analysis of recovered microspheres and microcapsules, average of two determinations.
e. Estimated 10.1% of WR 2721 recovered (neat) under similar hydrolysis conditions (pH 1).
f. Protection factor: % WR 2721 recovered (in microspheres or microcapsules ÷ % WR 2721 recovered (neat) under same conditions.
g. Microspheres.
h. Microcapsules.

TABLE VII. MICROSPHERE AND MICROCAPSULE ANALYSES AND HYDROLYSIS TEST RESULTS (pH 3)

| Run No.[a] | Theoretical[b] Payload % | Original[c] Assay % | After Test[d] Assay % | % WR 2721 Recovered |
|---|---|---|---|---|
| 1-10C[e] | 20.0 | 19.5 | 15.6 | 80.0 |
| 1-11C[e] | 20.0 | 18.9 | 16.4 | 86.8 |
| 1-14B[f] | 14.4 | 16.0 | 14.3 | 89.4 |
| 1-22[f] | 14.0 | 14.0 | 11.5 | 82.1 |
| 1-27[e] | 20.0 | 20.7 | 15.6 | 75.4 |

a. Composition given in Tables IV and V.
b. Gravimetric composition of melt prior to microsphere and microcapsule production.
c. Determined by HPLC analysis, average of two determinations.
d. Determined by HPLC analysis of recovered microspheres and microcapsules, average of two determinations.
e. Microspheres.
f. Microcapsules.

## D. Hydrolytic Stability of Unencapsulated WR 2721

Hydrolysis rate studies have been carried out on buffered solutions of WR 2721 at pH 7, 8, 9, 10, and 11. This work is an extension of the rate studies which were done at acidic pH during the first year of the program.[1,2]

In contrast to its susceptibility to rapidly hydrolyze in acidic solution at 37°C, WR 2721 was much more stable under alkaline conditions at the same temperature. It can be seen in the pH-rate profile (Figure 8) that the rate constant for the process is $<0.0002\ min^{-1}$ for the pH range 7-11. This corresponds to a half-life of >3 days. Calculated rates and half-lives are presented in Table IX.

TABLE VIII. PERFORMANCE DATA OF MOST PROMISING FORMULATIONS

| Matrix Composition[a] | Formulation Type | Run No. | Protection[b] Factor | 95% Release, min.[c] Tris Buffer | 95% Release, min.[c] Synthetic Intestinal Fluid |
|---|---|---|---|---|---|
| Stearic acid/paraffin wax (87.5/12.5) | microspheres | 1-10C | 8.8 | 30 | 13 |
| Stearic acid/paraffin wax (87.5/12.5) | " | 1-27 | 8.5 | - | 12 |
| Stearic acid/triglyceride (50/50) | " | 1-11C | 8.8 | 21 | 25 |
| Stearic acid/triglyceride (50/50) | microcapsules | 1-14C | 9.3 | 48 | - |
| Stearic acid/triglyceride (50/50) | " | 1-22 | 8.8 | - | 94 |

a. See Tables IV and V for details
b. See Appendix F.
c. See Discussion, Section V.C.

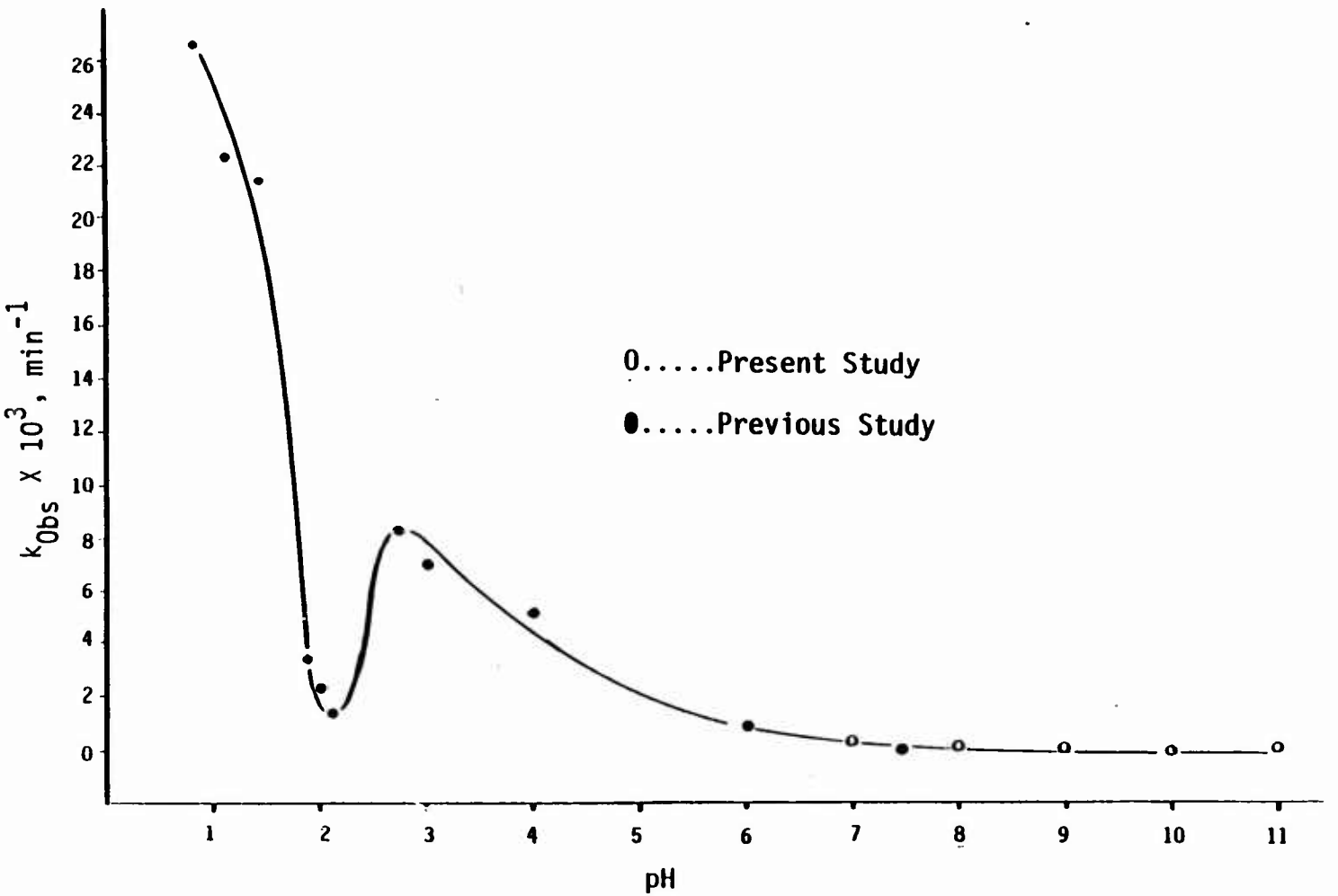


Figure 8. pH - Rate Profile for Hydrolysis of WR 2721 at 37°C

TABLE IX. RATES OF HYDROLYSIS OF UNENCAPSULATED WR 2721 AT BASIC pH

| pH[a] | $k_{obs} \times 10^3$, $min^{-1}$ | $t_{1/2}$, hour | Sampling Period, min | Data Points n[b] | Correlation[c] Coefficient |
|---|---|---|---|---|---|
| 7.01 | 0.359 | 32 | 120 | 8 | -0.977 |
| 8.03 | 0.153 | 75 | 150 | 6 | -0.867 |
| 9.02 | 0.0838 | 138 | 120 | 9 | -0.783 |
| 10.00 | -0.0787[d] | -- | 120 | 9 | +0.798 |
| 11.00 | 0.0979 | 118 | 120 | 9 | -0.895 |

a. Measured after deoxygenation step.

b. Number of data points used in calculation of $k_{obs}$.

c. For fit of regression of ln (absorbance) versus time data.

d. See discussion.

# V. DISCUSSION

## A. Microencapsulation

### 1. Microspheres

Based on the processing conditions established by the runs presented in Table III, a series of runs as presented in Table IV was conducted to prepare microspheres of various size ranges using triglyceride, fatty acid, fatty alcohol, and paraffin wax matrices. Assay of the microspheres indicated that little, if any, decomposition of the WR 2721 occurred during the encapsulation process. Runs 1-10C and 1-11C produced apparently satisfactory microspheres which showed good resistance to acid hydrolysis, and had desirable release rates. Efforts to reproduce some of the encapsulation runs gave mixed results due to mechanical problems. Duplicate runs were attempted of the two runs, 1-10C and 1-11C, (duplicate runs 1-24, 1-27 and 1-25, 1-28, respectively). Only in the case of Run 1-27 was it possible to closely reproduce process conditions. As a result, the encapsulation device has been modified to permit greater control of production parameters.

Payload of the microspheres was varied from 30 to 45 percent using milled WR 2721. At the 40 to 45 percent level, problems occurred during production, and the product microspheres failed the hydrolytic stability test. With the exception of Runs 1-36B and 1-40B attempts to prepare microspheres at the 30 and 35 percent payload level were unsuccessful. The cause(s) is not yet known.

### 2. Microcapsules

Microcapsules, in which a core of the active drug mixed with excipients is protected by a shell containing no active material, should give a dosage form of improved physical and chemical characteristics. Microcapsules (Runs 1-14B, 1-21, 1-22, and 1-26) were therefore prepared using stearic acid/triglyceride as the shell and core excipients. Runs 1-14B and 1-22 provided capsule samples which were resistant to acid hydrolysis.

Of the four encapsulation runs conducted, Run 1-22 was a repeat run using process conditions approximating those of Run 1-14B. Evaluation of these capsules indicated good product reproducibility based on hydrolytic stability and release-rate test data. In Runs 1-21 and 1-26, operating conditions were intentionally changed, as indicated in Table V, to determine if an improved product could be obtained. The hydrolytic stability results indicated an unsatisfactory product.

Encapsulation Run 1-39 was made using stearic acid and triglycerides in the shell and core formulations with a high percentage payload. Unsatisfactory capsules were obtained - most probably due to incomplete coating of the slightly thixotropic core. The formulation gave poor protection of the WR 2721 against acid hydrolysis.

B. Hydrolytic Stability of Encapsulated WR 2721

The five formulations found to be most resistant to aqueous acid solutions in the hydrolytic stability test (pH 1, 37°C, 90 minutes) included microspheres and microcapsules, and matrices of stearic acid/paraffin wax and stearic acid/triglyceride. Common to all was the payload level, ~20 percent. It was also observed that each of the same five formulations retained slightly less (~1-9 percent) of its payload when subjected to stability testing at pH 3. In addition, the formulations remained essentially intact during the test, as judged by appearance and by weight loss. It would appear that loss of WR 2721 is a diffusion process dependent upon one or more of the following variables.

1. Matrix composition
2. Uniformity of the matrix
3. Extent of ionization of WR 2721
4. Nature of solutes in the aqueous media which could aid transport across the matrix barrier.

The first two variables are controllable, therefore the ultimate extent of protection achieved will greatly depend upon how well the in vitro models mimic the animal system.

C. In Vitro Release Rates

In vitro release rates on the five most acid-resistant formulations are being determined by analysis of the recovered microspheres and the aqueous release media. Accountability for all tests is 100 ± 10 percent. Scatter is greater on tests conducted in synthetic intestinal fluid because the analytical method (OPA derivatization) is less precise. Using the analyses of recovered formulations, plots of ln (% recovered/100) versus time were found to be linear through $\geq$1.5 decades, and 95 percent release times were calculated using slopes of the linear regressions. When release was rapid and only two data points were obtained (15 and 30 minutes), linearity was assumed and the two points determined the line. At very rapid release only the 15 minute analysis showed remaining WR 2721, consequently the 95 percent release time was calculated from the following expressions.

$$\ln \frac{\% \text{ recovered}}{100} = -kt \qquad (1)$$

$$\% \text{ recovered} = \frac{100\ C_t}{C_o}$$

where $C_o$ = original assay
$C_t$ = assay at time t
$k$ = rate constant

$$t_{95\ \% \text{ release}} = -\ln \frac{0.05}{k} = \frac{3}{k} \qquad (2)$$

D. Current Good Laboratory Practice Regulation (CGLP) Adherence

During this annual report period, considerable efforts were undertaken in preparation to operate under CGLP as initially required under continuation of the contract; however, the contract has since been modified such that the CGLP requirement has been deleted.

E. Hydrolytic Stability of Unencapsulated WR 2721

The pH-rate profile for the hydrolysis of WR 2721 at 37°C is an exponential-like curve, increasing with increasing acidity and interrupted by a sharp minimum near pH 2. Similar profiles are exhibited by other phosphorothioates containing the 2-aminoethyl moiety.[11] This stabilization has been attributed to intramolecular complexation of the protonated amino group with an oxygen of the phosphate group. In contrast, WR 2721 exhibited no second smaller maximum in the basic pH range, although it is possible that a sharp deviation would have gone undetected had it occurred in an interval between data points. Note that the rate constant calculated for hydrolysis at pH 10 is a negative value (Table VIII). This apparent anomaly may be explained by 1) the very slow hydrolysis rate and 2) the analytical method used to monitor the reaction. It can be shown that, after addition of N-ethylmaleimide (NEM) reagent to an aliquot of the quenched reaction mixture, absorbance at 300 nm is proportional to the concentration of WR 2721, and a plot ln (absorbance) versus time yields the desired rate constant as its slope. This relationship does not hold at slow hydrolysis rates when 1) oxygen is present in the system causing the liberated thiol to be converted to its disulfide and/or 2) no allowance is made for the slow decomposition of the NEM reagent. A plot of ln (absorbance) versus time for the NEM reagent in the absence of WR 2721 (i.e., a "blank") allowed its hydrolysis rate constant to be estimated as $8.25 \times 10^{-5}$ $min^{-1}$ (25°C). This corresponds to a decrease of 0.001 absorbance units (a.u.) per 10 minutes when the total absorbance of the solution is about 0.5 a.u. This rate is actually faster than the rates of hydrolysis of WR 2721 at pH >8. It is believed that a small amount of oxygen remaining in the pH 10 reaction mixture combined with the correction for the blank was responsible for the anomalous value at pH 10.

As a check on the NEM analytical method, aliquots from the pH 11 experiment were removed at a 16-hour interval and analyzed for WR 2721 by HPLC (derivatization with o-phthalaldehyde). The rate constant calculated using this direct method of analysis was the same as that determined using the NEM procedure, within experimental error (see precision and accuracy of derivatization method in Analytical Procedures section of this report).

# VI. CONCLUSIONS

1. WR 2721 is hydrolytically unstable under acid conditions but stable under alkaline conditions.

2. WR 2721 can be successfully encapsulated as microspheres or as microcapsules. Additional encapsulation studies are required to optimize process parameters and to establish maximum payloads consistent with satisfactory end product properties.

3. Some of the formulations of WR 2721 showed good protection against acid hydrolysis and good release characteristics at pH 7.5.

4. Using a direct HPLC procedure, WR 2721 can be quantitated in microspheres, in microcapsules, and in some buffer solutions. Interferences present in synthetic intestinal fluid require that an alternate derivatization procedure be used to analyze for the drug in this medium.

# VII. RECOMMENDATIONS

1. Continue studies to increase WR 2721 payload in the microspheres and microcapsules.

2. Prepare additional WR 2721-containing microspheres and microcapsules using stearic acid, hydrogenated tallow glyceride, and paraffin wax to optimize process conditions.

3. Prepare microspheres and microcapsules with formulations containing various ratios of excipients to determine optimum formulations.

4. Determine stability of most promising microsphere or microcapsules at pH 1 and 3.

5. Determine release rates of most promising capsule samples at pH 7.5 in synthetic intestinal fluid.

6. Determine aging stability at 25 and 37°C for the most promising encapsulated samples.

7. Continue development of analytical methodology necessary for the blood analysis.

8. Determine the bioavailability of the most promising samples in vivo using the beagle dog as animal model.

9. Prepare and evaluate slow-release formulations of WR 2721.

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# APPENDIX A

# MATERIALS AND SUPPLIERS

TABLE A-1. MATERIALS

| Description | Source | Lot Number |
|---|---|---|
| S-2-(3-Aminopropylamino)-ethylphosphorothioic acid [WR 2721] | Walter Reed Army Institute of Research (WRAIR) | AU BJ 09506HJ-68-2 and |
| Atmul 84K [mono- and diglyceridies of edible oils and fats] | ICI United States, Inc. | 8103 |
| Emersol 6349 [stearic acid (70) food grade] | Emery Industries, Inc. | 19848 and 21375 |
| Emersol 6351 [stearic acid food grade] | Emery Industries, Inc. | 19848 |
| Grocol 55-E [stearic acid food grade] | A. Gross and Co. | 5-21459 |
| Emersol 6354 [lauric acid] | Emery Industries, Inc. | 19919 |
| 60N Food Grade HTG [hydrogenated tallow glycerides] | Acme-Hardesty Co., Inc. | E 880 |
| Grocol 600-E [hydrogenated tallow glycerides food grade] | A. Gross and Co. | 5-21459 |
| Paraffin N.F. | J. T. Baker Chemical Co. | 33,772 |
| Alfol 16 N.F. [cetyl alcohol] | Conoco Chemicals Co. | 16NF-14-L5 |
| Alfol 18 USP [stearyl alcohol] | Conoco Chemicals Co. | 0122-M65B-851 |
| Fully Refined Paraffin Wax 140/145 | Frank B. Ross Co., Inc. | 9273 |

# APPENDIX B

# HYDROLYTIC STABILITY OF WR 2721

## APPENDIX B

## HYDROLYTIC STABILITY OF WR 2721

The method described below is based on that reported by the National Formulary XIII entitled, "Release Tablets and Capsules - In Vitro Test Procedure," with minor modifications.

1.0 EQUIPMENT

1.1 Rotating bottle apparatus with constant temperature bath (37°C).

1.2 Support glassware, screens and filters.

1.2.1 90-mL bottles

1.2.2 80-mL beakers

1.2.3 4 dram vials

1.2.4 Vacuum desiccator

1.2.5 Stainless steel screen cloth, 105 μm

1.2.6 Vacuum pump

1.2.7 Analytical balance

1.2.8 45-mL repeater pipettes

2.0 TEST SOLUTIONS

2.1 Gastric Fluid, Simulated (Buffer solutions at pH levels of 1 and 3).

Buffer solutions are used as received. The solutions used should have a pH of about 1.0, and 3.0. The pH of the buffer solutions will be determined by pH meter before use.

3.0 PROCEDURE

3.1 All weights and measurements shall be recorded.

3.2 There shall be two sample per test (1.5 hour)

3.3 For each formulation to be tested, weigh two samples (~50 mg microspheres or ~75 mg microcapsules) on the analytical balance and place in two 90-mL bottles marked with the sample number.

3.4 Add 45 mL buffered solution (specific pH) (37°C) to each bottle. Record time T(0) when the last bottle is filled. This is the starting time for the test.

3.5 Cap bottles tightly and rotate in 37°C bath at 10-12 RPM.

3.6 Remove the sample(s) at 1.5 hour from T(0). Separate the microspheres or microcapsules using a fine mesh stainless screen. The filtrates are discarded. Discard the filtrates. Rinse the microspheres or microcapsules on the screen with water and place in a vacuum dissicator. Dry overnight at minimum pressure (vacuum pump). Place in clearly marked vials, flood with argon and retain in the freezer for analysis.

# APPENDIX C

## IN VITRO RELEASE-RATE ROTATING BOTTLE METHOD A

## APPENDIX C

## IN-VITRO RELEASE-RATE ROTATING BOTTLE METHOD A

The method described below is based on that reported by the National Formulary XIII entitled, "Release Tablets and Capsules - In Vitro Test Procedure," with minor modifications.

1.0 EQUIPMENT

1.1 Rotating bottle apparatus with constant temperature bath (37°C).

1.2 Support glassware, screens and filters.

1.2.1 90-mL bottles

1.2.2 80-mL beakers

1.2.3 4 dram vials

1.2.4 Vacuum desiccator

1.2.5 Stainless steel screen cloth, 105 μm

1.2.6 Vacuum pump

1.2.7 45-mL repeater pipette

1.2.8 Analytical balance

1.2.9 2000-mL volumetric flask

2.0 SYNTHETIC INTESTINAL SOLUTION OR TRIS BUFFER SOLUTION

2.1 All weights and measurements shall be recorded.

2.2 Synthetic Intestinal Solution

Mix: 13.6 g monobasic potassium phosphate
76.0 mL 1N sodium hydroxide

Dilute to 2000 mL with deionized water and adjust pH to 7.5 ± 0.1 with 1N NaOH if necessary. To each 100 mL solution to be used, add 1.0 g pancreatin just before use (this step is optional).

2.3 Tris (hydroxymethyl)aminoethane (Tris) Buffer

Mix: 50.0 mL of 0.2M solution of tris(hydroxymethyl)aminomethane (24.2 g in 100 mL).
41.4 mL of 0.2M HCl

Dilute to 200 mL with deionized water (pH 7.5 ± 0.1).

## 3.0 PROCEDURE

3.1 All weights and measurements shall be recorded.

3.2 There shall be seven sample times per formulation (at 1/4, 1/2, 1, 2, 3, 5, and 8 hours).

3.3 For each formulation to be tested, weigh seven samples (approx. 50 mg microspheres or approx. 75 mg microcapsules) on the analytical balance and place in seven 90 mL bottles marked with the sample number and time interval.

3.4 Beginning with the 8-hour sample(s), add 45 mL intestinal fluid or Tris Buffer solution (37°C) to each bottle. Record time T(0) when the last bottle is filled. This is the starting time for the test.

3.5 Cap bottles tightly and rotate in 37°C bath at 10-12 RPM.

3.6 Remove the first sample(s) at 1/4 hour from T(0). Separate the microspheres or microcapsules using a fine mesh stainless screen. Place solutions (~3 mL is retained) in clearly marked 4 dram vials and retain for analysis. The microspheres or microcapsules on the screen with water and place in a vacuum desiccator under vacuum (utilizing a vacuum pump) to dry overnight. Place in clearly marked vials, flood with argon and retain in the freezer for analysis.

3.7 Repeat Step 3.6 at the proper time intervals for the remaining samples.

3.8 The solutions from Step 3.6 may be frozen if necessary until analysis is undertaken.

# APPENDIX D

## HPLC PROCEDURE FOR ASSAYING WR 2721-CONTAINING MICROSPHERES OR MICROCAPSULES

## APPENDIX D

## HPLC PROCEDURE FOR ASSAYING WR 2721-CONTAINING MICROSPHERES AND MICROCAPSULES

A. Sample Preparation

1. Accurately weight out ~50 mg of microspheres or ~75 mg of microcapsules into a 4-dram vial.

2. Add 2 mL $CHCl_3$ to vial and allow 10-20 minutes for capsules to completely dissolve.

3. Add 10.0 mL of HPLC mobile phase.

4. Vigorously shake the capped 4-dram vial and allow the phases to separate. Inject a 20-µL aliquot of the upper (aqueous) layer onto the HPLC column.

B. HPLC Conditions

| | |
|---|---|
| Column: | Partisil 10 PAC (Whatman) 250 mm x 4.6 mm |
| Mobile phase: | 0.01M Phosphate buffer (pH 7.4) |
| Flow rate: | 1.5 mL/min |
| Detection: | UV at 205 nm |

C. Calibration

A solution of WR 2721 in the mobile phase is used as an external standard. A 5-mL aliquot of the standard is shaken with 1 mL of $CHCl_3$ and, after the phases have separated, a 20-µL aliquot of the upper (aqueous) layer is injected onto the HPLC column.

NOTE: This procedure has been developed to assay microcapsules or microspheres containing WR 2721 in a matrix of glycerides or fatty acids.

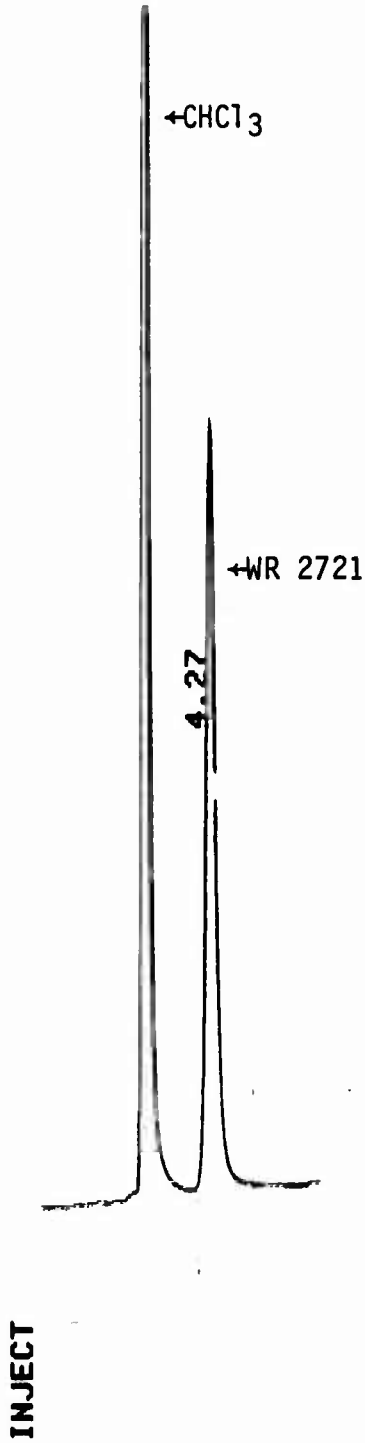



Figure D-1. Representative Chromatogram of Direct HPLC Assay of WR 2721-Containing Microcapsules

# APPENDIX E

## DERIVATIZATION PROCEDURE FOR ANALYSIS OF WR 2721

# APPENDIX E

## DERIVATIZATION PROCEDURE FOR ANALYSIS OF WR 2721

### HPLC Analysis

1. (a) Stock 300 mM $Na_xH_xPO_4$: 1 liter

   84 mM in $NaH_2PO_4$ (MW 138) 11.6 g
   216 mM in $Na_2HPO_4$ (MW 142) 30.67 g

   The pH of this stock solution should be 7.2 ± 0.1. Add 50 mL stock to each liter of mobile phase.

   (b) Solvent A - 15 mM $Na_xH_xPO_4$
   Solvent B - $CN_3CH/H_2O$ (55/45), 15 mM in $Na_xH_xPO_4$

2. Mobile Phase

   Use 50/50 A/B until further notice.

3. Detection

   Waters M420 Fluorescence Detector

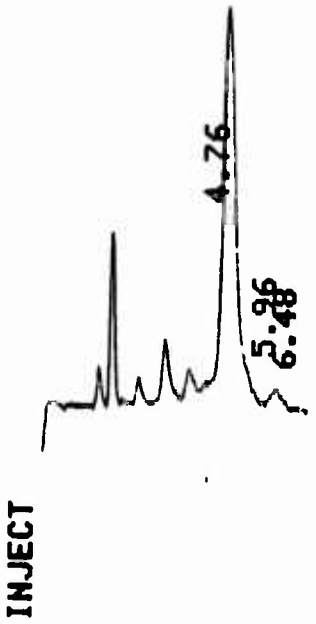
   excitation 338 nm (band pass)
   emission 425 nm (long pass)

### Reagents

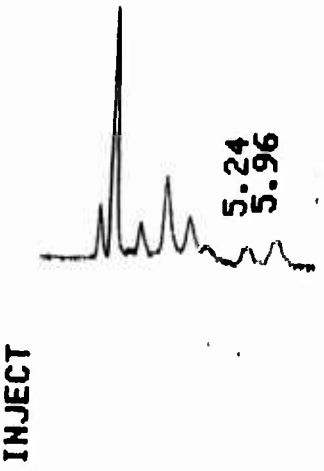
| | | Supplier | Catalog No. |
|---|---|---|---|
| 1. | 0.5M potassium borate (pH 10.5) Dissolve 30 grams of boric acid ($H_3BO_3$, MW 61.83) in 1 liter of water. Titrate to pH 10.4 with potassium hydroxide pellets. | Mallinckrodt | 2549 |
| 2. | o-Phthalaldehyde (MW 134.13) 20 mg/mL in methanol (74.5 µmol/mL) | Fisher | O-4241 |
| 3. | ethanethiol (MW 62.14) 10 µL/mL in methanol (135 µmol/mL) | Eastman | 109 4739 |

### Sample Preparation

1. Pipette 200 µL of WR 2721 sample into 10-mL volumetric flask.
2. Add 0.5 mL of borate buffer.
3. Add 1.0 mL methanol (HPLC grade).
4. Add 1.0 mL o-phthalaldehyde/ethanethiol combined reagent.
5. Let reaction mixture stand at room temperature for 1-2 minutes.
6. Dilute mixture with methanol to 10 mL and immediately inject 20 µL into HPLC.




Derivatized WR 2721 in synthetic intestinal fluid




Derivatized synthetic intestinal fluid

Figure E.1

# APPENDIX F

# METHOD FOR CALCULATION OF PROTECTION FACTOR

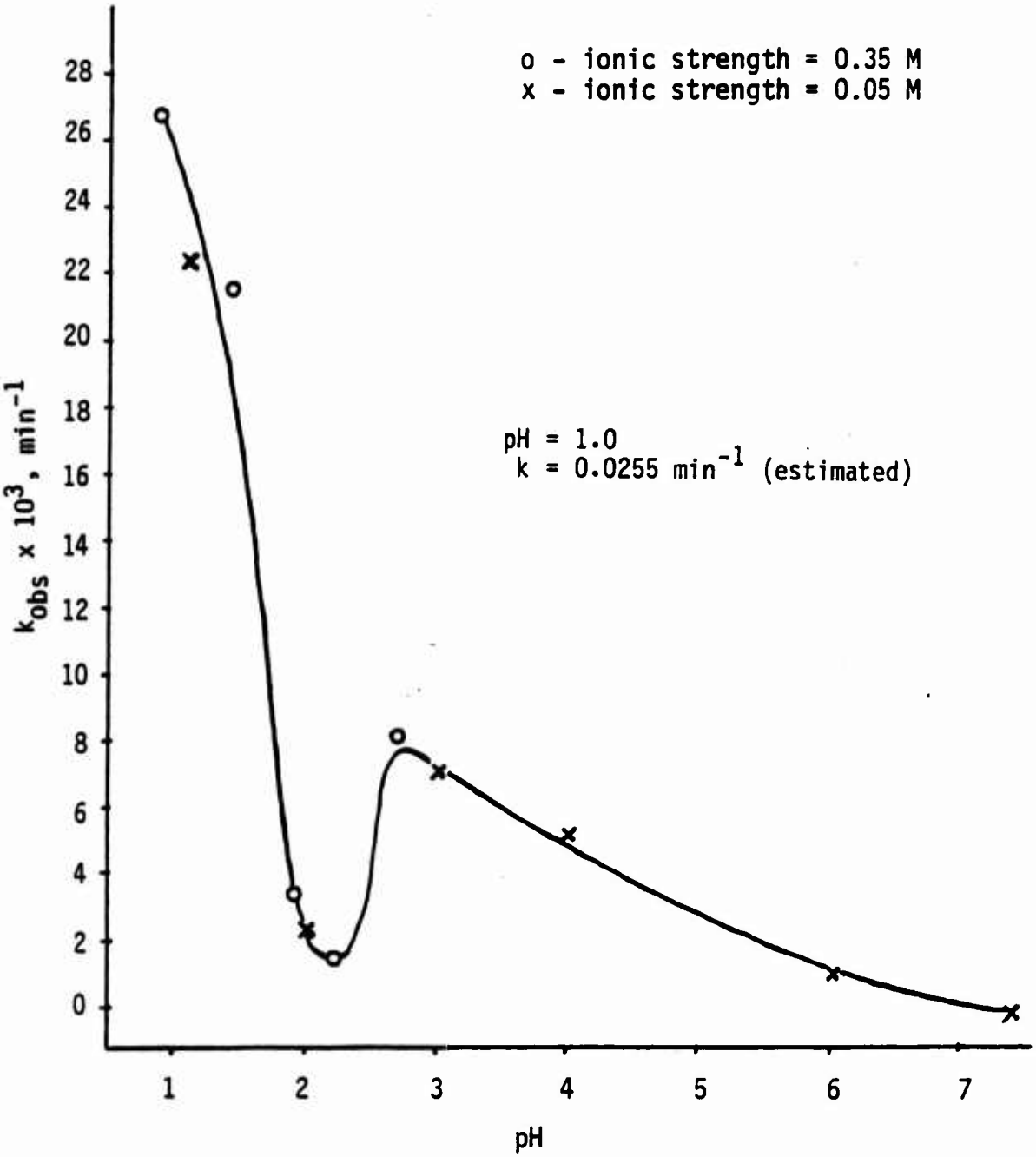



Figure F-1. pH-Rate Profile for Hydrolysis of WR 2721 at 37°C

# APPENDIX F
# CALCULATIONS

$C = C_0 e^{-kt}$ constant T

T = 37°C
k = 0.0255 $min^{-1}$ at pH = 1.0 (from curve)
t = 90 min

$$\frac{C}{C_0} = e^{(-0.0255)(90)} = e^{-2.295}$$

= 0.101 = fraction of original WR 2721 remaining after 90 min in pH 1.0 buffer at 37°C

= 10.1% (pH 1.0)

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# SUPPLEMENTARY

# INFORMATION

(6)

**DEPARTMENT OF THE ARMY**
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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REPLY TO ATTENTION OF: ERRATA

SGRD-RMI-S (70-1y) B080 528

31 JUL 1992

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-HDS/William Bush, Cameron Station, Bldg. 5, Alexandria, VA 22304-6145

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Development Command (USAMRDC), has reexamined the need for the limited distribution statement on technical reports for Contract No. DAMD17-80-C-0128. Request the limited distribution statement for AD Nos. ADB063672, [redacted], ADB094912, ADB094846, ADB109071, and ADB109038, be changed to "[redacted] distribution unlimited," and that copies of these reports be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Virginia Miller, DSN 343-7325.

ERRATA

CAREY O. LEVERETT
LTC, MS
Deputy Chief of Staff for
Information Management

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